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RESEARCH ARTICLE

High-Efficiency *Agrobacterium*-Mediated Transformation of Tobacco (*Nicotiana tabacum*)

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ABSTRACT: To improve *Agrobacterium*-mediated transformation of tobacco, factors influencing gene delivery, including genotype of the plant, bacterial strain, and *Agrobacterium* transformation procedure, were tested via direct somatic embryogenesis. Leaf tissue of three different tobacco genotypes (*Nicotiana tabacum* L. cvs. Samsun, and Xanthi, and *N. benthamiana*) were used as explant. Leaf explants were transformed using three *Agrobacterium tumefaciens* strains (EHA105, GV3101, and LBA4404) harboring the binary vector pCAMBIA1304 using three different types of transformation methods as named Agro-inoculation, Agro-infection and Agro-injection. Selection of hygromycin resistant shoots was conducted on MS medium containing 3.0 mgL^{-1} BAP and 0.2 mgL^{-1} IAA, 250 mgL^{-1} cefotaxime and 30 mgL^{-1} hygromycin. Hygromycin resistant shoots were then rooted on MS medium supplemented with 250 mgL^{-1} cefotaxime and 15 mgL^{-1} hygromycin. The results indicated that *A. tumefaciens* strain LBA4404 was more effective in gene delivery than EHA105 and GV3101 and Agro-infection method proved to be significantly better than two other methods. The highest transformation rate was obtained with the *Agrobacterium* strain LBA4404 and Agro-infection method with approximately 72.80%, 84.57%, and 93.33% for *N. benthamiana*, Samsun and Xanthi, respectively. Histochemical GUS assay confirmed the expression of *gus*A gene in putatively transformed plantlets. PCR and RT-PCR analysis using gene-specific primers confirmed the integration of the *gus*A and *hpt* genes and the expression of the *gus*A and *hpt* genes, respectively. Furthermore, Southern blot analysis confirmed stable integration of the *gus*A gene in selected T_0 transformants.

KEYWORDS: *Agrobacterium tumefaciens*, Direct somatic embryogenesis, Regeneration, Tobacco, Transformation

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; AS, acetosyringone; BAP, 6-benzylaminopurine; *gfp*, green fluorescent protein; *gus*A, β-glucuronidase; *hpt*, hygromycin phosphotransferase; IAA, indole-3-acetic acid; PGR, plant growth regulator; WT, wild type; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) of the Solanaceae family has been widely used as a model plant to understand biological processes and for functional genomics, proteomics, and metabolomics studies. *Agrobacterium*-mediated transformation of tobacco has become routine, and tobacco plants have been emerged

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as the leading plant platform for a variety of purposes, such as characterization of gene functions and production of recombinant biopharmaceuticals. Also, its regeneration is simply accomplished under in vitro conditions (36). The *in vitro* regeneration of plants is carried out by organogenesis or somatic embryogenesis. In contrast to organogenesis, somatic embryogenesis has some significant advantages including single-cell origin, the low frequency of mosaic plants and the production of a high number of regenerates (3). Somatic embryogenesis is a very valuable tool to investigate the biochemical, physiological and morphological events of zygotic embryogenesis in higher plants (13). The one of most important applications of somatic embryogenesis is the high propagation of plants through the embryogenic lines (20). Another application is in the production of plants with different levels of ploidy, via inducing of haploid and triploid embryos by cultivating anthers and endosperm, respectively (32). Also, somatic embryos can be used in synthetic seed technology due to success in inducing dormancy and therefore long-term storage (16). The somatic embryos as a source of protoplasts can be used in cell suspension cultures, offering a great potential for in vitro production of embryo metabolites (12). Finally, somatic embryogenesis has become a vital tool for gene transfer and production of genetically transformed plants (25).

Agrobacterium tumefaciens is a naturally occurring soil borne pathogenic bacterium that causes crown gall disease in dicotyledonous plants (3). The *Agrobacterium*-mediated transformation is an efficient and low-cost tool that exploits the natural ability of *A. tumefaciens* cells to transfer and integrate T-DNA into the host plant genome (27). The genetic transformation mediated by *Agrobacterium* has some important advantages than other transformation methods such as biolistic. The *Agrobacterium*-mediated plant transformation is a single-cell transformation system and not forming chimeras. As well as, the transfer of a single copy number of transgene results in fewer problems with transgene co-suppresion and instability (3). Efficiency of *Agrobacterium*-mediated transformation and delivery of T-DNA into plant cells is influenced by several factors, including genotype of the plant (10), type and age of explant (15, 27), strain of *A. tumefaciens* (24), the type of vector (29), bacterial cell density (19), pre-culture period (27), acetosyringone (AS) concentration (19), infection time (37), co-cultivation period (4), pH in co-cultivation medium (37), co-cultivation temperature (37), composition of culture medium, (27), and *Agrobacterium* infection methods (15).

Nowadays, there have been a large number of published reports on the *Agrobacterium*-mediated transformation of tobacco. Pathi et al. (25) developed a direct somatic embryogenesis method for four cultivars of tobacco by using 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). They transformed leaf explants using *Agrobacterium* strain LBA4404 and estimated the transformation efficiency more than 95%. In other investigation, the transformation efficiency of five *Agrobacterium* strains was evaluated in *Nicotiana tabacum* L. cultivar Samsun. The highest transformation rate (20%) was obtained with *Agrobacterium* strain LBA4404 (2). *Agrobacterium*-mediated transformation of wild tobaccos *N. debneyi*, *N. clevelandii*, and *N. glutinosa* were also conducted with *A. tumefaciens* strain EHA105 carrying the binary vector pBISN1 containing β-glucuronidase (*gus*A) reporter gene. The kanamycin-resistant plants were obtained from all three wild tobaccos at frequencies of 75.6% for *N. debneyi*, 25.0% for *N. clevelandii*, and 2.8% for *N. glutinosa* (5). The purpose of the present research is improvement of transformation efficiency of tobacco by investigation of several factors influencing the delivery of *Agrobacterium* T-DNA, including genotype of the plant, bacterial strain, and *Agrobacterium* infection procedure, using direct somatic embryogenesis from leaf discs as explant.

MATERIALS AND METHODS

Preparation of culture media and growth conditions

The MS salt solution (Murashige and Skoog, 1962) with B5 vitamins, 20 gL^{-1} sucrose, 8 gL^{-1} plant-agar was used in all the experiments. All plant growth regulators (PGR) were added before autoclaving at 121°C for 20 min. The pH of the media was adjusted to 5.8 by 1 M NaOH or 1 M HCl before autoclaving. The culture media were incubated under culture room conditions including 25 ± 2°C with 20-25 µmol m^{-2} s^{-1} irradiance provided by cool fluorescent lamps, a photoperiod of 16 h and 55% relative humidity.

Seed sterilization and germination

Seeds of three different genotypes of tobacco (*N. tabacum* L. cvs. Samsun and Xanthi, and *N.*

benthamiana) were surface-sterilized by immersion in 100% ethanol for 1 min and 50% commercial bleach (3% sodium hypochlorite) for 15 min (35). The seeds were thoroughly washed with sterile distilled water and then blotted dry on Whatman filter paper. The sterilized seeds were germinated on MS medium and maintained under culture room conditions.

Induction of direct somatic embryogenesis and plant regeneration

First pairs of fully grown leaves from two month-old plants were trimmed in 1 × 1 cm pieces and placed with the adaxial side up onto culture medium under sterile conditions. To induce direct somatic embryogenesis, leaf explants were placed on MS medium with different concentrations of IAA (0.0, 0.1, 0.2, and 0.3 mgL^{-1}) and BAP (0.0, 1.0, 2.0, and 3.0 mgL^{-1}) for determination the best concentration producing somatic embryos. For regeneration of plants, the explants bearing cotyledonary stage embryos were transferred to MS basal medium supplemented with 30 gL^{-1} sucrose. After two weeks of growth, embryo-derived shoots were separated and cultured individually on MS basal medium supplemented with 30 gL^{-1} sucrose to induce root elongation. All cultures were incubated under culture room conditions, as mentioned above. The plantlets with a good-developed root system and 8-10 leaves were transferred to plastic pots containing peat moss and sand (1:1) and grown under greenhouse conditions at 25 ± 2°C with a photoperiod of 16 h. The pots were enveloped in polyethylene bags and then bags were removed after 7 days.

Agrobacterium strains and preparation of bacterial cultures

Three *A. tumefaciens* strains (EHA105, GV3101, and LBA4404), harboring the binary vector pCAMBIA1304, carrying the β-glucuronidase (*gus*A) and hygromycin phosphotransferase (*hpt*) genes under the control CaMV35S promoter and *nos* terminator were used for tobacco transformation. The *gus*A gene contains an intron in its coding region to inhibit GUS expression in *Agrobacterium* cells and confirmation of its activity in the plant cells. The *A. tumefaciens* strains were grown on YEP agar medium supplemented with 50 mgL^{-1} of Kanamycin and 25 mgL^{-1} Rifampicin. A single colony of each *Agrobacterium* strain was separately inoculated into 15 ml of YEP medium containing the same antibiotics and incubated at 28°C on an orbital shaker at 150 rpm for 48 h. An aliquot of 500 µl of each bacterial suspension was discretely added into 50 ml YEP medium with the additional antibiotics and grown overnight at 28°C. The bacterial cells were harvested from the overnight grown culture by centrifugation at 3000 g for 15 min. The bacterial pellets were re-suspended in liquid co-cultivation medium with 200 µM AS to a final OD_{600} of 0.6 (35). The re-suspended bacterial cells were shaken at 150 rpm at 28°C for 60 min before use.

Agrobacterium-mediated transformation

The leaf explants and leaves were pre-cultured for 48 h and then infected to different *A. tumefaciens* strains harboring pCAMBIA1304 binary vector. Three different types of transformation methods were performed as named Agro-inoculation, Agro-infection and Agro-injection. In the Agro-inoculation method, the prepared *Agrobacterium* inoculums were separately transferred to sterile Petri dishes and pre-cultured leaf explants were inoculated for 30 min with shaking the Petri dishes every 10 min. In the second type of transformation, pre-cultured leaves were trimmed in 1 × 1 cm pieces by infected scalpels to different *Agrobacterium* strains. In the Agro-injection method, the *Agrobacterium* inoculums were separately injected into the pre-cultured leaves by a 1 ml needleless syringe. The *Agrobacterium*-treated explants and leaves were blotted on sterile filter paper and placed with the adaxial side up onto co-cultivation agar medium overlaid with a single piece of sterile filter paper for 72 h in the dark at 25 ± 2°C. After co-cultivation period, the explants were first washed by 250 mgL^{-1} cefotaxime for three times and then transferred to selection agar medium supplemented with 250 mgL^{-1} cefotaxime and 30 mgL^{-1} hygromycin. The cultures were sub-cultured every 10 days to three times. Following a four weeks' culture period, hygromycin-resistant somatic embryos were developed. The explants were transferred to regeneration medium supplemented with 250 mgL^{-1} cefotaxime and 15 mgL^{-1} hygromycin. The explants were planted on fresh regeneration medium every 2 week. After four weeks of growth, hygromycin-resistant shoots were separated and cultured individually on rooting medium supplemented with the same antibiotics and 10 gL^{-1} sucrose. A root system was established within 2 weeks and then transformed plantlets were transferred to soil.

GUS histochemical analysis

The histochemical analysis of *gus*A gene expression was performed on the putatively transformed plantlets as indicated by the established method (11). The putatively transformed and wild type (WT) plantlets were incubated for 72 h at 37 °C in a buffer containing 50 mM $NaPO_4$ (pH 7.2), 20 mM EDTA (pH 8.0), 0.1% Triton X-100, 500 mgL^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and 20% methanol. After X-gluc staining, the plantlets were cleared by 70% ethanol to remove the chlorophyll. GUS expression was visually observed and photographed by using Canon PC1057 camera.

gDNA and total RNA extraction and PCR analysis

gDNA and total RNA were extracted from the leaves collected from the putatively transformed plantlets and WT plants using CTAB reagent (6). First strand cDNA was then synthesized from 3 µg of total RNA treated with RNase-free DNase I (Fermentas, Germany), using RevertAidTM M-MuLV Reverse Transcriptase (Fermentas, Germany) and Oligo $(dT)_{18}$ (Fermentas, Germany) as the initiation primer. Reverse transcription was carried out at 42°C for 1 h and terminated by heating to 70°C for 10 min. The integration and expression of *gus*A and *hpt* genes were determined by PCR and reverse transcription (RT)-PCR, respectively. PCR and RT-PCR were performed using specific *gus*A, *hpt* and *virG* primers (Table 1). The PCR reaction was carried out in a thermal cycler (Techne, UK) programmed for 35 cycles; conditions for each cycle being denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The plasmid pCAMBIA1304 and Ti plasmid, and genomic DNA of WT plants were used as positive and negative controls, respectively. The amplified products were resolved on a 1.2% (w/v) agarose gel.

Southern blot analysis

Southern blot analysis was performed to find the copy number of the *gus*A gene in transformed PCR positive plantlets using Dig DNA Labeling and Detection Kit (Roche, Switzerland) according to the manufacturer's instructions. Twenty µg of DNA from transformed PCR positive plants, WT plants, and 5 µg of pCAMBIA1304 plasmid DNA were digested overnight with *Eco*RI at 37 °C. The binary vector pCAMBIA1304 and DNA from WT plantlets served as positive and negative controls, respectively. The digested DNA samples were resolved on a 1 % (w/v) agarose gel, then depurinated, denatured, neutralized and blotted onto nitrocellulose membrane (Hybond-N+, Amersham, England). The membrane was hybridized with Dig-labeled amplicon of *gus*A gene (1182 bp) as probe. The hybridized membrane was first washed three times, 10 min each, with low stringency buffer (1×SSC: 0.3 M sodium chloride; 0.03 M sodium citrate; 0.1% (w/v) SDS) at room temperature. Afterwards, the membrane is washed twice, 30 min each, with high stringency buffer (0.1×SSC: 0.03 M sodium chloride, 0.003 M sodium citrate, 0.1% (w/v) SDS). The hybridized membrane was subjected to Anti-Digoxigenin-alkaline phosphatase solution and then nitroblue tetrazolium (NBT) solution was added to membrane.

Table 1. Primers used in this study

Primer	Sequence (5' → 3')
gusA	gusF: ATACCGAAAGGTTGGGCAGG; gusR: ATAACGGTTCAGGCACAGCA
hpt	hygF: CTATTTCTTTGCCCTCGGAC; hygR: AAGCCTGAACTCACCGCGAC
virg	virgF: CATTTTCGTCATCCGCGGTC; virgR: CAGTTGAGGGCTTGACGGAT

Statistical analysis

Number of embryos were recorded after 6 weeks of culture. Each treatment was repeated twice and each treatment consisted of 3 replicate culture Petri dishes, each containing four leaf explants. In *Agrobacterium*-mediated transformation of different tobacco genotypes, each treatment consisted of three replicates with 15 explants per Petri dish. The results were analyzed statistically by SPSS ver. 16 (SPSS Inc., Chicago, IL, USA). Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the Duncan multiple range test calculated at the confidence level of $P < 0.05$. Variability of the data was expressed as mean ± standard error (SE).

RESULTS

Direct somatic embryogenesis and plant regeneration

The various combinations of BAP and IAA, added to basal MS medium (2% sucrose) were analyzed for the

induction of direct somatic embryogenesis from leaf disc explants of three genotypes of tobacco (*N. tabacum* L. cvs. Samsun and Xanthi and *N. benthamiana*). After three days of subculture, the edges of leaf explants became bulged and folded, and all explants became swollen and enlarged in size within a week of culture. During the second week of culture, most of the somatic embryos were formed on the leaf discs at different developmental stages such as globular, heart, torpedo and cotyledonary shaped stages (Fig. 1A–H). The leaf discs cultured on basal MS medium without PGRs as controls, did not produced somatic embryos or callus, and turned brown and became necrotic within 2-3 weeks. But explants which were cultured only on BAP or IAA induced organogenesis or callus and roots, respectively (data not shown). The formation of somatic embryos was emerged on all media tested and all tobacco genotypes examined had 100% somatic embryo induction efficiency. However, the percentage of embryogenesis was significantly influenced by the concentration of the PGRs used in the medium. In the lower concentrations of both the hormones, the minimum number of somatic embryos per explant was produced. Further increase in the concentrations of BAP (3.0 mgL^{-1}) and IAA (0.3 mgL^{-1}), was also let to a decrease in the number of somatic embryos per explant, indicating the negative effect of BAP and IAA on somatic embryogenesis induction. In all the combinations of BAP and IAA tested, the maximum number of somatic embryos per explant were obtained in the MS medium supplemented with 3.0 mgL^{-1} BAP and 0.2 mgL^{-1} IAA (Table 2). An average 167.00, 105.92 and 118.25 somatic embryos were formed per 1 × 1 cm leaf segment of *N. benthamiana*, Samsun and Xanthi, respectively. There were no morphological aberrations and the frequency of

Table 2. Effect of different concentrations of PGRs (BAP and IAA) used for direct somatic embryogenesis of different genotypes of *N. tabacum*

Genotype	PGR Con. (mgL^{-1})		Frequency of explants with somatic embryo formation (%)	No of somatic embryos per explant (Mean ± SE)*
	BAP	IAA		
N. benthamiana	1.0	0.1	100	80.88 ± 1.66^{ghi}
	1.0	0.2	100	68.88 ± 4.55^{ij}
	1.0	0.3	100	86.13 ± 1.37^{gh}
	2.0	0.1	100	115.44 ± 2.14^{cd}
	2.0	0.2	100	147.92 ± 9.68^{b}
	2.0	0.3	100	102.97 ± 2.02^{def}
	3.0	0.1	100	154.72 ± 9.73^{b}
	3.0	0.2	100	167.00 ± 1.13^{a}
	3.0	0.3	100	149.61 ± 7.49^{b}
N. tabacum cv. Samsun	1.0	0.1	100	92.22 ± 5.26^{fg}
	1.0	0.2	100	85.58 ± 9.24^{gh}
	1.0	0.3	100	65.17 ± 3.20^{j}
	2.0	0.1	100	90.42 ± 5.89^{fgh}
	2.0	0.2	100	83.33 ± 1.72^{gh}
	2.0	0.3	100	83.58 ± 7.99^{gh}
	3.0	0.1	100	93.14 ± 9.28^{efg}
	3.0	0.2	100	105.92 ± 4.43^{cde}
	3.0	0.3	100	80.25 ± 4.84^{ghi}
N. tabacum cv. Xanthi	1.0	0.1	100	50.50 ± 2.84^{k}
	1.0	0.2	100	82.92 ± 6.04^{gh}
	1.0	0.3	100	43.83 ± 4.64^{k}
	2.0	0.1	100	67.75 ± 6.91^{ij}
	2.0	0.2	100	94.17 ± 1.45^{efg}
	2.0	0.3	100	89.38 ± 5.41^{gh}
	3.0	0.1	100	84.92 ± 6.69^{gh}
	3.0	0.2	100	118.25 ± 3.12^{c}
	3.0	0.3	100	77.25 ± 5.99^{hij}

* The results of twice experiments each with three replicates, each replicate containing four leaf explants. Different letters in a column indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

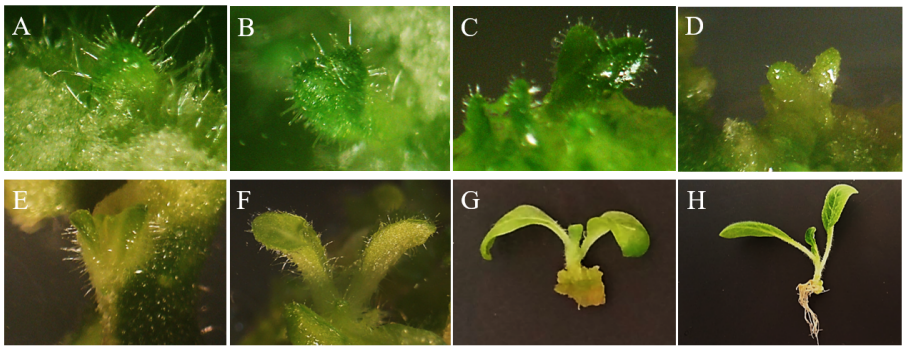

Figure 1. Various stages of direct somatic embryogenesis of *N. tabacum* cv. Xanthi. (A) Fused globular stage embryos; (B) early heart shape stage; (C) heart shaped stage; (D) early torpedo stage; (E) torpedo stage; (F) cotyledonary stage; (G) plantlet; (H) plantlet with a developing root system. Pictures were taken by Canon PC1057 camera.

somatic embryos formation per explant was affected by genotype. For plant regeneration, somatic embryos at the late cotyledonary stage were transferred to hormones free MS medium (3% sucrose) for root growth. When a root system had developed and 8 to 10 leaves had formed, the plants were transferred to soil and grown to maturity under greenhouse conditions. The plantlets were successfully acclimatized with approximately 80.0%, 91.7%, and 100% survival for *N. benthamiana*, Samsun and Xanthi, respectively. There were no morphological differences between regenerated plants and seed-derived controls.

Agrobacterium-mediated transformation

Various factors affecting the transformation efficiency, including the *Agrobacterium* strain, genotype, and type of *Agrobacterium* infection procedure, were evaluated in this work. The other factors influencing *Agrobacterium*-mediated transformation such as pre-culture period (48 h), AS concentration (200 μM), *Agrobacterium* bacterial concentration (OD_{600}: 0.6), *Agrobacterium* infection time (30 min), the days of co-cultivation (72 h), co-cultivation medium pH (5.4), and temperature in co-cultivation (25℃) were considered as cited by Wang (2006). Three different *A. tumefaciens* strains (EHA105, GV3101, and LBA4404) were used in transformation of different tobacco genotypes. *Agrobacterium* strains that are useful for plant transformation are defined by their chromosomal background and resident Ti plasmid. LBA4404 is an octopine strain with Ach5 chromosomal background carrying pAL4404 as virulence plasmid (7), GV3101 is a nopaline strain with a C58 chromosomal background. It contains pMP90 as virulence plasmid (8) and EHA105 contains a disarmed version of the L,L-Succinamopine type supervirulent Ti plasmid pEHA105 (pTiBo542DT-DNA) (9).

The pre-cultured leaf explants and leaves were infected to different *A. tumefaciens* strains harboring pCAMBIA1304 binary vector by using three different types of transformation procedure, Agro-inoculation, Agro-infection and Agro-injection. The putative transgenic somatic embryos that thrived on the selection medium developed, through the normal stages of embryogenesis (globular, heart-shape, torpedo-shaped, and cotyledonary stages). These embryos germinated into plantlets in the hygromycin supplemented regeneration media with proper rotting and morphological characters. The well rooted plantlets were further transferred to pots and exhibited healthy growth, normal flowering and seed set (Fig. 2A-F). The transformation frequency in tobacco was significantly influenced by the genotype, bacterial strain, and *Agrobacterium* transformation procedure (Table 3). The highest transformation rate was obtained with the *Agrobacterium* strain LBA4404 and Agro-infection method with approximately 72.80%, 84.57%, and 93.33% for *N. benthamiana*, Samsun and Xanthi, respectively.

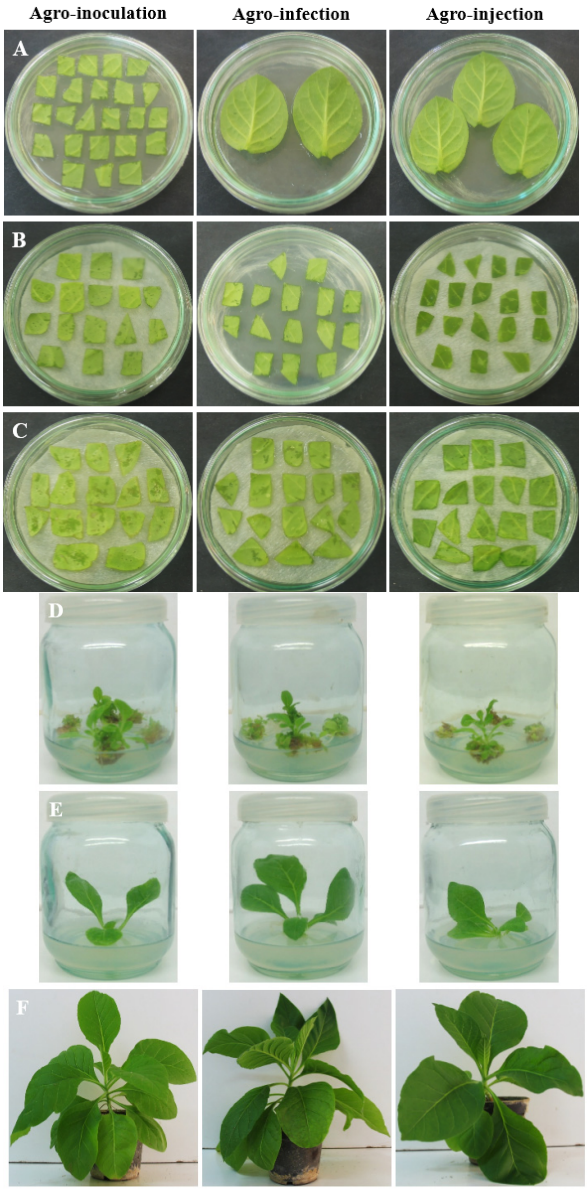

Figure 2. *Agrobacterium*-mediated transformation of *N. tabacum* cv. Xanthi by *Agrobacterium* strain LBA4404. (A) Pre-culture of explants; (B) co-cultivation; (C) selection; (D) maturation of cotyledonary stage embryos; (E) elongation and rooting; (F) hardening in green house.

Table 3. *Agrobacterium*-mediated transformation of different tobacco genotypes using leaf explants

Genotype	*A. tumefaciens* strain	Transformation method	Experiment	Total No. of explants	No. of explants producing Hyg^R plantlets	No. of explants producing GUS^+ plantlets (No. of Hyg^R plantlets assayed)	Transformation frequency (%)[a]
N. benthamiana	EHA105	Agro-inoculation	BEM1	45	30	20 (28)	47.55 ± 4.70^{efgh}
		Agro-infection	BEM2	45	33	20 (30)	49.33 ± 6.10^{efg}
		Agro-injection	BEM3	45	30	21 (29)	48.50 ± 5.31^{efgh}
	GV3101	Agro-inoculation	BGM1	45	6	4 (6)	8.94 ± 4.47^{no}
		Agro-infection	BGM2	45	9	7 (9)	15.49 ± 2.16^{mn}
		Agro-injection	BGM3	45	17	10 (17)	22.35 ± 2.22^{klm}
	LBA4404	Agro-inoculation	BLM1	45	33	23 (30)	56.43 ± 5.03^{de}
		Agro-infection	BLM2	45	38	26 (30)	72.80 ± 3.72^{bc}
		Agro-injection	BLM3	45	29	17 (27)	40.53 ± 4.22^{fghij}
N. tabacum cv. Samsun	EHA105	Agro-inoculation	SEM1	45	32	22 (29)	54.20 ± 7.18^{def}
		Agro-infection	SEM2	45	42	26 (30)	81.10 ± 6.03^{ab}
		Agro-injection	SEM3	45	18	13 (18)	28.79 ± 4.50^{jkl}
	GV3101	Agro-inoculation	SGM1	45	23	15 (23)	33.46 ± 3.87^{ijk}
		Agro-infection	SGM2	45	34	20 (30)	50.07 ± 1.61^{efg}
		Agro-injection	SGM3	45	23	16 (23)	35.69 ± 5.92^{hij}
	LBA4404	Agro-inoculation	SLM1	45	38	23 (30)	64.80 ± 5.33^{cd}
		Agro-infection	SLM2	45	44	26 (30)	84.57 ± 2.92^{ab}
		Agro-injection	SLM3	45	27	18 (26)	41.32 ± 4.07^{fghij}
N. tabacum cv. Xanthi	EHA105	Agro-inoculation	XEM1	45	30	22 (28)	52.53 ± 5.73^{defg}
		Agro-infection	XEM2	45	42	26 (30)	80.67 ± 1.59^{ab}
		Agro-injection	XEM3	45	24	18 (24)	39.97 ± 3.88^{ghij}
	GV3101	Agro-inoculation	XGM1	45	27	19 (26)	43.53 ± 3.78^{efghi}
		Agro-infection	XGM2	45	23	16 (23)	35.58 ± 4.39^{hij}
		Agro-injection	XGM3	45	12	8 (12)	17.82 ± 2.21^{lmn}
	LBA4404	Agro-inoculation	XLM1	45	41	26 (30)	78.57 ± 3.45^{b}
		Agro-infection	XLM2	45	45	28 (30)	93.33 ± 3.33^{a}
		Agro-injection	XLM3	45	29	20 (28)	46.03 ± 3.17^{efghi}

The values represent the means ± SE, each from three replicates with 15 explants per Petri dish. Means followed by different letters are significantly different at $p < 0.05$ with Duncan's multiple range test.

[a] Transformation frequency (%) = (No. of explants producing GUS^+ plantlets/ No. of Hyg^R plantlets assayed) × (No. of explants producing Hyg^R plantlets/ Total No. of explants) × 100

GUS histochemical assay

Expression of the *gus*A-intron gene is a reliable indicator of plant transformation, since the gene only can express efficiently in plant cells but not in *Agrobacterium* (34). The GUS staining was particularly very strong in plantlets transformed by LBA4404 strain, but no GUS activity was detected in plant transformed with native *A. tumefaciens* without expression vector pCAMBIA1304 as a negative control (Fig. 3).

PCR, RT-PCR and Southern blot analysis

To confirm the presence of the *gus*A and *hpt* genes in transformed plants, PCR analysis was conducted on putative transformants, along with plants transformed with native *A. tumefaciens* without expression vector pCAMBIA1304 (as negative controls), pCAMBIA1304 plasmid DNA (as positive control for *gus*A and *hpt* genes), and Ti plasmid DNA (as positive control for *Vir*G). The integration of the *gus*A and *hpt* genes in the

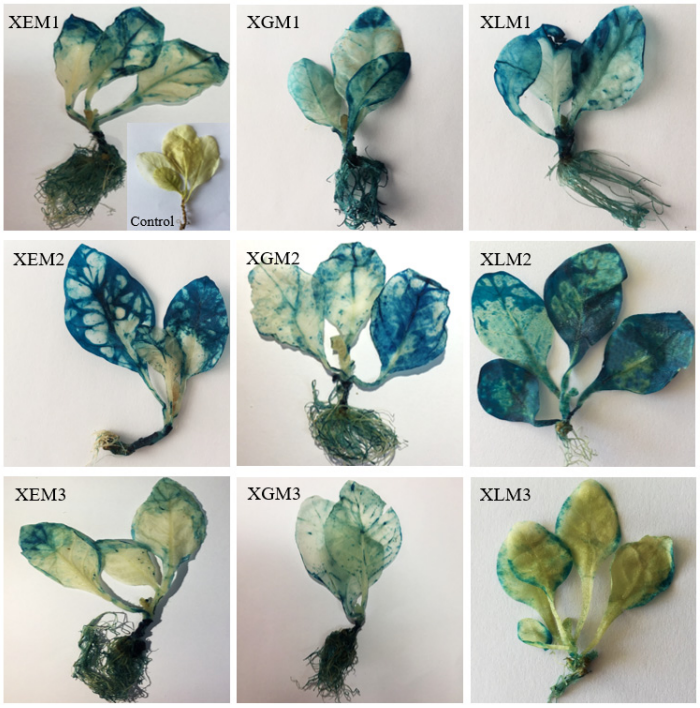

Figure 3. Histological GUS assay in *N. tabacum* cv. Xanthi transformed with different *Agrobacterium* strains by various transformation methods.

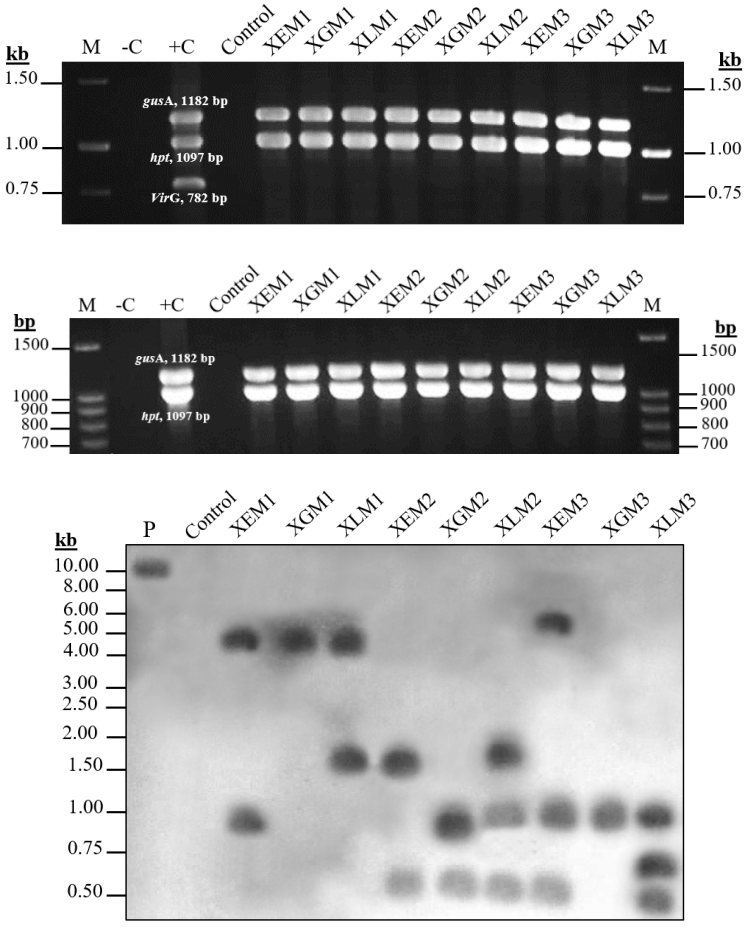

Figure 4. PCR, RT-PCR and southern blot analysis of the putative transformants of *N. tabacum* cv. Xanthi. (A) PCR analysis of the putative transformants using *gus*A, *hpt*, and *Vir*G genes specific primers. (B) RT-PCR analysis of the putative transformants using *gus*A and *hpt* genes specific primers. M, 1 kb or 100 bp DNA ladder; -C, negative control without template in PCR reactions; +C, positive controls (expression vector pCAMBIA1304 and Ti plasmid) in PCR reactions; Control, plant transformed with native *A. tumefaciens* without expression vector pCAMBIA1304. (C) Southern blot analysis of the putative transformants using *gus*A gene as probe. P, plasmid pCAMBIA1304 as positive control.

genome of putative transformants was confirmed by the presence of an amplified fragment of 1182 bp and 1097 bp, respectively (Fig. 4A). Additionally, no band corresponding to *Vir*G gene (782 bp) was detected in putative transformants, showing amplification of *gus*A and *hpt* genes from genome of transformed plants. The expression of *gus*A and *hpt* genes was also confirmed by RT-PCR analysis (Fig. 4B). Amplification of these fragments was not observed in non-transformed plants.

Southern blot analysis of the putative transformants confirmed the integration of the *gus*A gene into the plant genome (Fig. 4C). Genomic DNA was digested with *Eco*R I (cutting the T-DNA at a unique site) and probed with the *gus*A gene coding region. No hybridization signals could be detected for the plants transformed with native *A. tumefaciens* without expression vector pCAMBIA1304 as negative controls. Southern hybridization pattern of selected transgenic plants confirmed single as well as multiple gene insertion. Studies have shown that it is desirable to have a single gene insertion in transgenic plants as multiple copies of T-DNA adversely influence the expression of the introduce gene (30).

DISCUSSION

In vitro propagation via somatic embryogenesis has become an essential tool for genetic transformation and regeneration of large numbers of transgenic plants (25). The leaf segments are the most reliable sources to generate plants through direct somatic embryogenesis. Histological observations have indicated that somatic embryos are formed directly from epidermal or subepidermal cells of the leaf explants without any vascular connection with the maternal tissue (31). In our experiments, the somatic embryos were successfully formed from leaf explants in all tobacco genotypes within 2 weeks. The embryogenesis process from somatic tissue was operated without an intervening callus phase and progressed through the stages typical for zygotic embryogenesis. Differences between cultivars in the efficiency and frequency of somatic embryos production were observed and may be explained by the presence of various levels of endogenous phytohormones, particularly cytokinins, in different genotypes might influence their response to somatic embryogenesis (3). Variations in somatic embryogenesis have been known to happen due to a number of different factors, such as basal medium (26), explant source (17) and genotype (28). As well, the type and age of explants has an impact on somatic embryogenesis (26). The effect of genotype on somatic embryogenic has been clearly revealed. For instance, in cassava (*Manihot esculenta*), out of the eight cultivars studied, somatic embryogenesis could only be induced in five (28).

A well-balanced combination of cytokinins and auxins lead to the embryogenic response of leaf explants. In this work, optimal embryo formation takes placed on medium supplemented with 3.0 mgL^{-1} BAP and 0.2 mgL^{-1} IAA. Auxins and cytokinins are critical agents for cell differentiation and specification during embryogenesis (22). It was suggested that exogenous PGRs influence both the establishment of cell polarity and the subsequent cellular processes leading to the formation and development of normal somatic embryos (3). Auxins promote swelling explants within one week of culture and somatic embryos started to differentiate from surfaces of explants. Cytokinins are known to stimulate cells and are crucial for a high frequency of somatic embryos (18). It was well documented that auxin plays a role in the formation of apical–basal axis of the embryo by influencing endogenous IAA, which is a critical event in plant embryogenesis (22). In carrot cells, application of exogenous 2,4-dichlorophenoxyacetic acid (2,4-D) induced somatic embryo formation via accumulation of large amounts of endogenous IAA (21).

Agrobacterium-mediated transformation is the preferred technique for genetic modification in most plant species because of its easy accessibility, stable gene expression, and tendency to produce low- or single-copy insertion of the transgene (3). A number of factors involved in genetic transformation greatly influence the overall gene transfer efficiency. In this study, the effects of several independent factors influencing the efficiency of *A. tumefaciens*-mediated transformation of tobacco were investigated. These factors included genotype of the plant, bacterial strain, and *Agrobacterium* transformation procedure. *A. tumefaciens* differs in its ability to infect different species or genotypes of plants. Generally, the specificity of genotype is related to the cell physiological conditions, such as physiological reaction of cell after wounding, concentration of internal PGRs of cell and structure of cell wall (10). Therefore, it was likely that for these reasons *N. tabacum* cv. Xanthi demonstrated better response compared to other genotypes, lending to highest transformation efficiency.

Similarly, genotypic influence on transformation efficiency has been displayed in chickpea (*Cicer arietinum* L.) (10). Duan et al. (5) reported that transformation frequency in tobacco is species dependent. They transformed three different wild tobacco species by *A. tumefaciens* strain EHA105 with various transformation frequencies of 75.6% for *N. debneyi*, 25.0% for *N. clevelandii*, and 2.8% for *N. glutinosa*.

A. tumefaciens strains play an important role in the plant transformation process. The virulence of *Agrobacterium* strains differs widely among host plant species depending on the interaction between the *Agrobacterium* strain and host plant (38). The different interacting proteins in plants were reported to be involved in T-DNA and virulence protein transfer, cytoplasm trafficking, nuclear targeting, T-DNA integration, stability and expression, and defense responses (40). In *Arabidopsis*, *rat* mutants resistant to *Agrobacterium*-mediated transformation failed to express genes encoding proteins implicated in chromatin structural and remodeling, and T-DNA transfer and integration (39). Repression the programmed cell death response has also revealed to increase the frequency of transformation. For instance, expressing the animal antiapoptosis genes in banana suspension cells, improved the frequency of transformation by 90% (14). In our experiments, *Agrobacterium* strains differ in their ability to transform different tobacco genotypes and LBA4404 seems to be the best strain for a suitable transformation system in tobacco. The use of *Agrobacterium* strain LBA4404 in transformation of Xanthi cultivar, resulted in higher transformation rates. The LBA4404 strain has been used successfully in the transformation of various plant species (2, 15).

Agrobacterium-mediated genetic transformation includes different methods such as Agro-inoculation, vacuum infiltration, sonication, and *in-planta* transformation techniques (1, 10, 15). The sonication treatment acts as an abiotic stress in plants and causes to increase cell permeability. The micro-wounds or fissures formed after sonication improve accessibility of *Agrobacterium* to target cells (15). Treatment of target tissues by ultrasound for brief periods in the presence of *Agrobacterium* lead to a 100- to 400-fold increase in transient GUS expression (33). Three different types of transformation methods were used in present research. Transformation via Agro-infection method proved to be the most efficient for the different tobacco genotypes with a higher transformation percentage compared with lower levels of transformation when the Agro-inoculation and Agro-injection methods were used. Contrary to Agro-inoculation and Agro-injection methods, *Agrobacterium* infection are confined to the edges of leaf explants in Agro-infection procedure that lead to formation putatively transformed plantlets in wounded edges of explants. The wounding cause to initiation of active cell division, improved binding of *Agrobacterium* to the newly synthesized cell wall at the wounded sites, production of v*ir*-inducing compounds by the metabolically active cells, and finally increase of transformation frequency (40).

CONCLUSION

The present study has developed an efficient method for the improvement of transformation frequency of three different genotypes of tobacco using *Agrobacterium*. A number of factors which are important in the delivery of *Agrobacterium* T-DNA including genotype of the plant, bacterial strain, and *Agrobacterium* infection procedure were evaluated. The present results demonstrated the feasibility and effectiveness of *Agrobacterium* strain LBA4404 and Agro-infection method for gene delivery in different tobacco genotypes.

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بهبود تراریزش گیاه توتون به واسطه اگروباکتریوم از طریق جنین‌زایی سوماتیکی مستقیم

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چکیده

به منظور بهبود تراریزش گیاه توتون به واسطه اگروباکتریوم از طریق جنین‌زایی سوماتیکی مستقیم، عوامل موثر بر انتقال ژن از قبیل ژنوتیپ گیاه، سویه اگروباکتری و روش تراریزش گیاهی مورد بررسی قرار گرفتند. به همین منظور بافت برگی سه ژنوتیپ مختلف توتون شامل *Nicotiana benthamiana* و واریته‌های Samsun و Xanthi از گونه *N. tabacum* به عنوان ریزنمونه مورد استفاده قرار گرفت. ریزنمونه‌های برگی با استفاده از سه سویه مختلف *Agrobacterium tumefaciens* (EHA105، GV3101 و LBA4404) حامل ناقل دوگانه pCAMBIA1304 و با سه روش متفاوت تراریختگی تحت عناوین Agro-inoculation، Agro-infection و Agro-injection تراریزش شدند. گزینش نوساقه‌های مقاوم به هیگرومایسین روی محیط MS حاوی BAP ۰/۳ mgL^{-1}، IAA ۰/۲ mgL^{-1}، ۲۵۰ mgL^{-1} سفوتاکسیم و ۳۰ mgL^{-1} هیگرومایسین انجام شد. سپس نوساقه‌های مقاوم روی محیط MS حاوی ۲۵۰ mgL^{-1} سفوتاکسیم و ۱۵ mgL^{-1} هیگرومایسین ریشه‌دار شدند. نتایج نشان داد که سویه LBA4404 نسبت به سویه‌های EHA105 و GV3101 و روش Agro-infection نسبت به دو روش دیگر در انتقال ژن به طور معنی‌داری موثرتر هستند. بالاترین میزان تراریزش در ژنوتیپ‌های *N. benthamiana* و واریته‌های Samsun و Xanthi با استفاده از سویه LBA4404 و روش Agro-infection به ترتیب معادل ٪۷۲/۸۰، ٪ ۸۴/۵۷ و ٪۹۳/۳۳ بدست آمد. آزمون هیستوشیمیایی GUS و آنالیزهای PCR و RT-PCR، دخول و بیان ژن‌های *gus*A و *hpt* را در گیاهچه‌های تراریخته تایید نمودند. همچنین، آنالیز ساترن بلات، وجود ژن *gus*A را در گیاهان تراریخته نسل T_0 به اثبات رساند.

کلمات کلیدی: تراریزش، توتون، جنین‌زایی سوماتیکی مستقیم، *Agrobacterium tumefaciens*، باززایی