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Full Length Research Paper

Studies on plant regeneration and transformation efficiency of *Agrobacterium* mediated transformation using neomycin phosphotransferase II (*nptII*) and glucuronidase (GUS) as a reporter gene

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Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, is the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. We have standardized the tissue culture media for the regeneration and transformation with the vector LBA 4404 (pCAMBIA 2301), so that in future, this system may be exploited for the expression of antibody fragment (single chain variable fragment) in plants (plantibody). The transformed green shoots tested positive for neomycin phosphotransferase II (*npt*II) gene and glucuronidase (GUS) were screened, rooted on MS medium and subsequently hardened to harvest seeds. The transformation frequency of *Agrobacterium* (LBA 4404) with the binary vector pCAMBIA 2301 on the basis of GUS resistance was found to be 2.9%.

Key words: *Agrobacterium,* transformation, *Nicotiana tabacum*, tobacco, transformation frequency.

INTRODUCTION

Recent advances in plant biotechnology have provided a powerful tool for the construction of genetically improved transgenic plants (Gasser and Fraley, 1989). Molecular manipulation is becoming very important in plant improvement. The chimeric gene construct can stably be introduced into the plant genome by different techniques such as electroporation, biolistics and protoplast fusion (Walden and Schell, 1990). For dicots, *Agrobacterium* mediated transfer is an efficient, cost effective method for gene delivery from *Agrobacterium* to a nuclear genome of the plant cells with high transformation frequency, less DNA rearrangement and silencing than direct DNA transfer (Gallie, 1998). The use of reporter gene simplifies the expression of the gene in transgenic plants and is widely used as a scorable marker. Normally, the glucuronidase (GUS) gene is absent in plant tissues and no detectable background was obtained in most of the higher plant cells. The sensitivity of the assay makes this marker useful for verification of transformation (Jefferson et al., 1987).

In this study, the *Agrobacterium*-mediated transformation for *Nicotiana tabacum* cv Xanthi (Turkish) from callus was optimized using GUS as a reporter. The effect of parameters such as bacterial concentration, pre-culture period, co-cultivation and immersion time were estimated. These parameters are known to influence the transformation efficiency and the optimized conditions are host species dependent. This study was carried out to generate an efficient regeneration and transformation

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Abbreviations. GUS, Glucuronidase; **BAP,** 6-benzylamino purine; **NAA,** α-naphthaleneacetic acid; **MS,** Murashige and Skoog; ***npt*II,** neomycin phosphotransferase II; **IBA,** indole- 3-butyric acid; **O.D,** optical density; **scFv,** single chain variable fragment, **YEM,** yeast extract mannitol; **RM,** regeneration medium; **CTAB,** cetyl trimethylammonium bromide; **LB,** Luria Bertani.

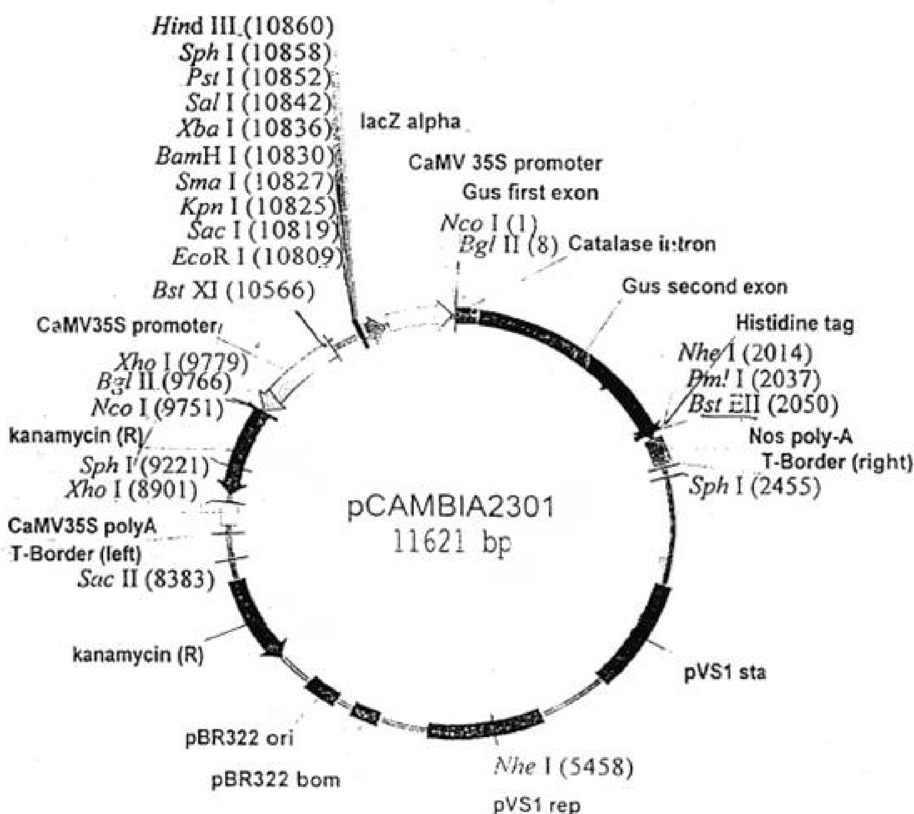

Figure 1. Vector map of pCAMBIA 2301.

protocol with this variety so that in future this system may be exploited for expression of Single Chain Variable Fragment (scFv) for production of recombinant anti-bodies.

MATERIALS AND METHODS

Seeds of genotype of *N. tabacum* cv. Xanthi (Turkish) were obtained from the Central Tobacco Research Institute, Rajamundry, Andra Pradesh (ICAR), India.

Callusing and organogenesis from leaf discs of *N. tabacum* cv. Xanthi

Leaf discs, used in the present study were obtained from 6 - 7 week old plants, grown aseptically, cut from their edges to remove midrib and made into square piece of approximate 1 cm^2. Thirty leaf discs were aseptically cut and transferred to MS medium (Murashige and Skoog, 1962) containing varying concentration of 6-benzylamino purine (BAP) (0.75 to 1.5 mg/l) and α-naphthaleneacetic acid (NAA) (0.05 to 0.15 mg/l). The leaf discs were incubated under cool fluorescent light (3000 lux) with 16 h photoperiod. Callusing and shoot regeneration response were observed after one month of culture. Shoot regeneration or callusing frequency (%) was calculated by dividing the number of explants which callused or formed shoots by the total number of explants placed in the particular combination. The regenerated shoots were excised with some callus still attached and transferred to elongation medium (MS containing 0.125 to 1.0 mg/l BAP and 0.125 to 1.0 mg/l kinetin). The elongated shoots were cut, transferred to ½ MS medium with different concentration of indole- 3- butyric acid (IBA) (0.25 to 1.0mg/l). Observations for rooting and numbers of root per shoots were taken after 3 weeks of culture. The *in-vitro* rooted plantlets were hardened in Hoagland's solution for 4 weeks and transferred to pots containing sterilized soil:sand:farm yard manure (2:1:1) for hardening in glass house. Within 5 weeks, the plants were transferred to plantation pots and grown to maturity.

Source of *Agrobacterium* strain and plasmid

Agrobacterium strain (LBA 4404) and pCAMBIA 2301 (11621 bp) with 2 kb GUS gene and neomycin phosphotransferase II (*npt*II) selectable marker under the control of CaMV 35S promoter and nos terminator were provided by Professor Akhilesh Tyagi, Department of Plant Molecular Biology, South Campus, New Delhi, India (Figure 1).

Studies on pCAMBIA based vector

The plasmid pCAMBIA 2301 was transformed into the *Agrobacterium* by heat shock method of transformation (Sambrook et al., 1989) with slight modification. The single colony of transformed *Agrobacterium* was inoculated in 50 ml of yeast extract mannitol (YEM) medium containing 50 µg/ml each of kanamycin and rifampicin. The culture was incubated at 28°C at 120 rpm in an orbital

shaker up to late log phase. The plasmid was isolated by alkaline lysis method (Maniatis et al., 1989) and confirmed by restriction digestion with *Nhe*I and *Eco*RI (Table 2). Colony polymerase chain reaction (PCR) was performed by inoculating single colony of *Agrobacterium* in 25 µl of double distilled water and boiled for 2 min, and then placed on ice. 2 µl of this was used as template DNA. 10 mM of dNTPs, 1.5 U of taq polymerase, 1 x assay buffer and 40 ng of *npt* II specific primer set were used in PCR reaction. Samples underwent 94°C initial denaturation for 5 min, 37 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min and final extension of 72°C for 7 min. The amplified product was analyzed in 1% agarose gel and used as positive control.

Standardization of transformation protocol

Preparation of *Agrobacterium* culture

Primary culture of *Agrobacterium* was prepared by inoculating single colony of *Agrobacterium* in 20 ml YEM medium (with 50 µg/ml each of kanamycin and rifampicin) incubated at 28°C at 120 rpm for 28 h. The secondary culture was initiated by inoculating 1 ml of primary culture in 100 ml YEM without antibiotics, grown at 28°C till $O.D._{600}$ reached 0.5 to 0.6 and centrifuged at 5000 rpm for 15 min. The pellet was dissolved in the regeneration medium so as to dilute it to an $O.D._{600}$ = 0.1 - 0.3. This *Agrobacterium* suspension was used for standardization of infection and co-cultivation period for leaf discs at different time interval.

Transformation

Leaves from 7 - 8 week old plants were cut into 1 cm^2 and inoculated into liquid preculture medium (MS + 1.0 mg/l BAP + 0.1 mg/l NAA + 3% sucrose) for 24 h at 120 rpm. The leaf discs were placed in *Agrobacterium* suspension (O.D. = 0.1-0.3) for 10 - 30 min, blotted dry on sterile filter paper and co-cultivated at 28°C in co-cultivation media (MS + 1.0 mg/l BAP + 0.1 mg/l NAA + 3% sucrose) at 120 rpm for 24 h under diffused light at 24 - 25°C. The leaf discs were washed thrice at 120 rpm for 30 min in washing media (MS + 1.0 mg/l BAP +0.1 mg/l NAA + 300 mg/l cefotaxime). The explants were blotted dry and placed on regeneration medium (RM1) medium (RM + 300 mg/l cefotaxime + 10 mg/l kanamycin). Plates were incubated under cool fluorescent light (3000 lux), 16 h photoperiod for 10 days and were transferred to S1 selection medium (RM + 25 mg/l Kanamycin + 250 mg/l cefotaxime). After 3 weeks of cultivation the green shoots were separated and cultured on S2 selection medium (RM medium + 35 mg/l kanamycin + 250 mg/l cefotaxime) for 3 weeks. The surviving green shoots were subjected to two more passage of selection on S3 and S4 selection medium (MS + 50 mg/l kanamycin + cefotaxime 200 mg/l). The percent transformation frequency was calculated by dividing the number of explants surviving the four selection cycles by total number of explants co-cultivated. Elongated shoots were placed in S5 medium (1/2 M.S + 0.25 mg/l +IBA) for rooting of differentiated shoots.

Confirmation of the presence of transgene in putatively transformed rooted shoots

Histochemical GUS Assay

The transient expression was checked by histochemical assay (Jefferson, 1987). The transformed explants were incubated (two or three days after the co-cultivation) in X-Gluc solution for 24 h. One explant was randomly taken from each co-cultivated plate. A total of 60 transformed explants (30 from each of the two consecutive co-cultivated experiments) and untransformed plants (used as negative control) were incubated in X-Gluc. Stained explants were seen under stereo-zoom microscope and stored in 70% ethanol.

Selection of transformed plants through PCR:

Total genomic DNA was isolated from putative transformed and untransformed shoots using cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Primer set used for amplification of 786 bp fragment of the *npt*II gene was *npt*IIF, CGTCAAGAAGG CGATAGAAGG and *npt*II R,TGGGGATTGAACAAGATGGATT. PCR was carried out in 25 µl of reaction containing 50 ng of template DNA, 10 mM of dNTPs, 1.5 U of Taq polymerase, 1 x assay buffer and 40 ng of *npt* II specific primer set. PCR was performed with initial denaturation of 94°C for 5 min, 37 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 2 min and final extension of 72°C for 7 min and electrophoresed on 1.2% agarose gel.

RESULTS

Plant regeneration studies from leaf discs *of N. tabacum* cv. Xanthi.

Success of *Agrobacterium* mediated transformation and expression of any gene depends upon callusing and regeneration frequency, which helps in recovering large number of transformants. Leaf discs are most frequently used explants for *N. tabacum* transformation because they are easy to establish and yield many transformed plants (Horsch et al., 1985). The hormone combination 1.0 mg/l BAP and 0.1 mg/l NAA was used for callusing and shoot regeneration with maximum 73.3% of shoot regeneration and was found to be significant ($p \leq 0.05$) (Table 1) (Figure 2). The regenerated callus were sub-cultured at 4 - 5 weeks interval in the same regeneration medium and transferred to 0.25 mg/l BAP. Elongated differentiated shoots were separated after 3 weeks of culture and transferred to rooting media. Rooting initiated within 10 days and became profuse after 4 weeks of the culture. Highest number of roots per explants (19.7) with 86.6% of rooting was observed in ½ MS containing 0.5 mg/l IBA. 28 explants were transferred to the Hoagland medium, 23 acclimatized with profuse rooting (survival 82.2%), transferred to glass house and seeds were harvested.

Studies on pCAMBIA 2301

Transformation of plasmid pCAMBIA 2301 to A. tumifaciens (LBA 4404)

Plasmid DNA was mobilized into competent cells of *Agrobacterium* (prepared with treatment of chilled 10 mM Tris) by freeze thaw method of transformation and incubated at 28°C for 2 days; 50 - 60 colonies were selected on Luria Bertani (LB) broth (LB) plates containing 50 µg/ml kanamycin. The plasmid was isolated, band of 3444 bp and 8177 bp and a single band of 11621 bp were obtained on restriction digestion with *Nhe*I and *Eco*R1

Table 1. Effect of BAP and NAA on callus and shoot induction frequency of leaf disc of *Nicotiana tabacum*.

Concentration of BAP and NAA	No. of explants	No. of explants producing callus	Callus induction frequency (%)	No. of explant producing shoot	Shoot induction frequency (%)
0B 0N	30	0	0.0	0	0.0
0.75B 0.05N	30	18	60.0	8	26.6
1.0B 0.05N	30	15	50.0	5	16.6
1.25B 0.05N	30	17	56.7	7	23.3
1.50B 0.05N	30	20	66.7	8	26.6
0.75B 0.075N	30	21	70.8	10	33.3
1.0B 0.075N	30	19	63.3	12	40.0
1.25B 0.075N	30	16	53.3	9	30.0
1.50B 0.075N	30	18	60.0	10	33.0
0.75B 0.1N	30	23	76.7	11	36.7
1.0B 0.1N	30	26	86.7	22	73.3
1.25 B 0.1N	30	20	66.7	16	53.3
1.50B 0.1N	30	19	63.3	15	50.0
0.75B 0.125N	30	22	73.3	6	20.0
1.0B 0.125N	30	20	66.7	13	43.3
1.25B 0.125N	30	18	60.0	11	36.7
1.5B 0.125N	30	16	53.3	7	23.3
0.75B 0.15N	30	18	60.0	ND	–
1.0B 0.15N	30	19	63.3	4	133
1.25B 0.15N	30	17	56.7	3	10.0
1.50B 0.15N	30	15	50.0	5	16.7
S.Em.±	–	–	0.6	–	0.5
CD at 5%	–	–	1.8	–	1.3

*B, BAP; N, NAA.

Table 2. Restriction digestion mixture.

Component	Reaction mixture for *Nhe* I	Reaction mixture for *Eco*RI
DNA	10 µl (3µg)	12 µl (3 µg)
Nhe I (10U/ µl)	0.3 µl	_
*Eco*RI (20U/ µl)	_	0.25 µl
Buffer(10X)	2 µl	2 µl
Sterile ddw	7.7 µl	5.75 µl
Total	20 µl	20 µl

(Figure 3). Colony PCR gave the amplification of 786 bp of *npt*II gene (as predicted by DNA star software) when the amplified products were resolved in 1.2% (w/v) agarose gel. This LBA 4404 harboring pCAMB1A 2301 was used as a positive control for transformation studies.

Transformation experiments with *Nicotiana tabaccum* cv. Xanthi using *Agrobacterium* strain LBA4404 (pCAMBIA 2301)

Transformation of leaf discs were carried out at different time interval (10, 20, 30 min) and transformation frequency was recorded by counting the number of kanamycin resistant cultures obtained after three selection cycles and subculturing on the same concentration of kanamycin for 15 - 20 days each on medium containing 25, 35 and 50 mg/l kanamycin. The O.D. 0.3 and infection time of 30 min was found to be significant at $p \leq 0.05$ with maximum transformation frequency of 3.8% and used for transformation to obtain high transformants. 23 out of 60 explants (30 from each of two consecutive co-cultivation experiments) were found to be GUS positive and the transient expression frequency was found to be 38.3%. Five independent transformations were carried out at this infection of O.D 0.3 and incubation time of 30 min. During the process of the transformed leaf discs continued to grow vigorously to produce callus, whereas the untransformed ones failed to form callus, eventually bleached and became necrotic within 3 weeks of culture. 25 kanamycin resistant shooting cultures were obtained from 640 explants co-culture from different co-cultivation experiments after four cycles of selection (Table 3). The transformation frequency on the basis of kanamycin resistance was found to be 3.9% (calculated by dividing the total number of kanamycin resistant shoots surviving the S4 selection cycle by the

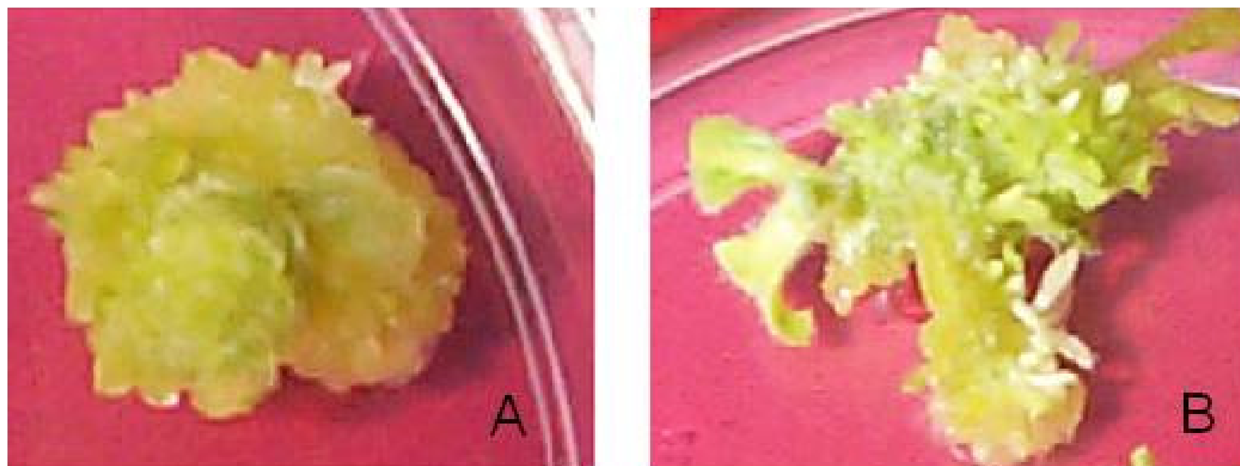

Figure 2. Callusing and shoot regeneration from leaf disc of *Nicotiana tabacum* in MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA**.** (A). Callus formation (B). Multiple shooting.

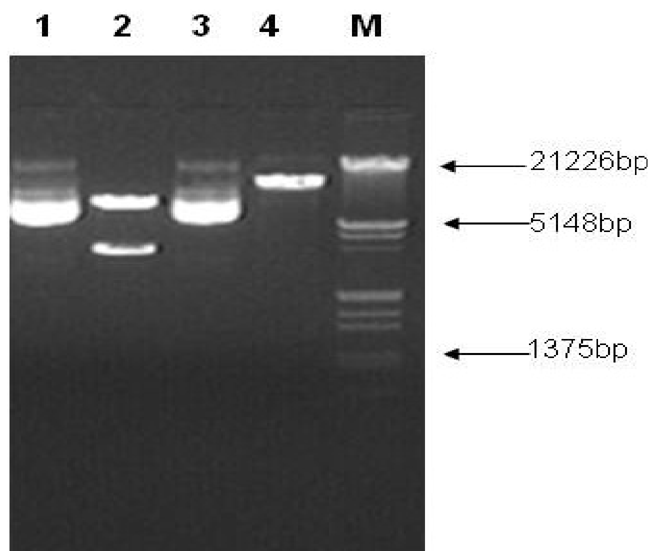

Figure 3. Restriction digestion of pCAMBIA 2301 isolated from transformed *Agrobacterium* LBA 4404M: λDNA EcoRI/Hind III double digest. L1: pCAMBIA 2301 (undigested); L2: pCAMBIA 2301 digested with *Nhe*I; L3: pCAMBIA 2301 (undigested); L4: pCAMBIA 2301 digested with *EcoR*I.

number of explants co-cultivated).

Confirmation of the putative transgenic lines

Histochemical GUS staining

25 shoots obtained after four cycles of selection were checked for the presence of β–glucuronidase enzyme. 19 were found to be positive showing localized blue spots, not uniform while untransformed ones were GUS negative (Figure 4). The GUS expression frequency was found to be 2.9% (calculated by dividing the number of explants showing GUS expression by total number of explants co-cultivated).

Genetic analysis of transgenic plants

Agrobacterium mediated transformation results in random integration of T-DNA in plant cell genome and thus a single transgenic plant can have multiple integration events of T-DNA. Although scoring the number of integration events in transgenic plants by southern hybridization is a routine protocol, it is time consuming and also tedious. Detection using PCR is a simple method for the identification of the transgene.

Genomic DNA of 11 plants (randomly selected) shown to be positive for GUS were taken and PCR amplified with *npt*II specific primer. The amplification of 786 bp compared with the positive control (plasmid) was observed in all GUS positive transformed shoots and no band was observed in the untransformed plants when analyzed on 1.5% agarose gel (Figure 5). All GUS positive shoots were also found to be positive for *npt*II. The plants putative transgenic were grown to maturity in the glasshouse to harvest seeds.

DISCUSSION

Efficient regeneration and transformation are major prerequisites for the development of suitable expression system. Therefore, prior to an experiment, it would be appropriate to have a standardized protocol to maximize the results. The hormonal combination of 1.0 mg/l BAP and 0.1 mg/l NAA was found to be best for the regeneration through the callus and the absorbance of 0.3 with infection time of 30 min was found to be most suitable

Table 3. Transformation frequency of *Nicotiana tabacum* at O.D 0.3 and 30 min infection time with LBA 4404 (pCAMBIA 2301).

Vector	Experim ent	No. of explants co-cultivated	No. of explants survived after selection cycle				Total no. of kanamycin resistant shoots
			S1 Selection cycle (a)	S2 Selection cycle (b)	S3 Selection cycle (c)	S4 subculture (c)	
pCAMBIA 2301	Control	20	0	0	0	0	
	1	150	101	56	19	8	
	2	150	91	41	13	7	
	3	120	73	39	8	4	
	4	100	55	23	5	2	
	5	120	69	24	7	4	
Total		640	389	183	52	25	25
Survival (%)			60.8	28.6	8.1	3.9	3.9

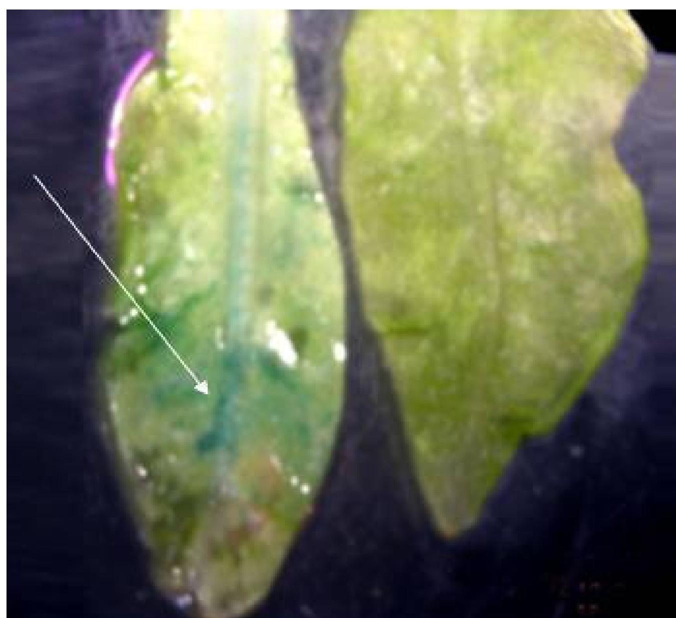

Figure 4. Histochemical GUS assay of transformed and control plant. Transformed (Left), control (right).

for the transformation. The transformation freq-uency reported for the transformation through the LBA 4404 (pCAMBIA 2301) on the basis of kanamycin resistance in this variety was 3.9%.

Stable plant transformation requires a considerable investment in time before the expressed proteins can be analyzed. In contrast, transient gene expression systems are rapid, flexible and straightforward. The transient expression assays described ensure that most errors and technical problems with gene expression can be identified and resolved before making stable transfor-mants (Kapila et al., 1996). It was found that none of the control plants gave positive result with the X-Gluc indicating the absence of any endogeneous GUS activity, whereas the transformed ones showed the presence of the blue spot indicative of the transient expression found to be 38.3%.

The transformation frequency with respect to GUS expression was found to be 2.9%. The positive lines were genetically analyzed and integration of the gene into the plant genome was confirmed by PCR (Li et al., 2006). The PCR amplification clearly depicted a 786 bp band and none in the untransformed plants. The GUS positive plants were found to be positive for the presence of transgene, therefore the transformation frequency for this variety was found to be 2.9%. Initial studies were done

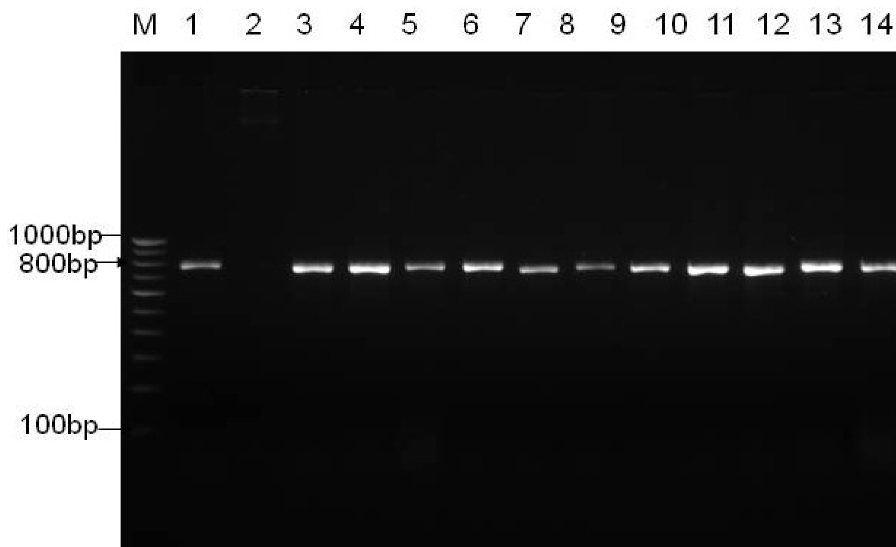

Figure 5. PCR amplification of the putative transgenic plants with *npt* II specific primers. Lane M: 100 bp Ladder; Lane 1: positive control; Lane 2: PCR amplicon from untransformed plant; Lane 3-14: PCR amplicon from transformed plant co-cultivated with LBA 4404 (pCAMBIA 230).

with the *Agrobacterium* LBA 4404 (pCAMBIA 2301) so that a system may be established in the authors laboratory for the expression of recombinant antibody fragment (scFv) in *Nicotiana* (our further study). In summary, the transformation and regeneration system described here is simple and effective, allowing routine introduction of gene into *N. tabacum* cv Xanthi (Turkish). The efficiency achieved with our protocol may produce hundreds of independent lines of transgenic tobacco within a six month period.

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