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Research Article

Expression and Inheritance of GUS Gene in Transgenic Tobacco Plants

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Abstract: *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf discs was performed with plant transformation vectors of pBI 121 and pGus-Int. Both carry the neomycin phosphotransferase II (*npt II*) gene and, unlike the pBI 121, pGus-Int carries a portable plant intron inside the beta-glucuronidase (GUS) gene. In addition to the integration of the marker genes into the genomes of primary transformants, R1 and R2 progenies were confirmed by polymerase chain reaction (PCR) and Southern blot analyses. Histochemical and fluorometric analyses were also performed to determine the activity of the GUS gene. Segregation of progenies on kanamycin-resistance trait was also shown as evidence for the transformation. The analyses showed that the transgenes were transmitted to subsequent generations in a Mendelian manner and intron sequences enhanced the expression of the GUS gene by 25-30% in some transformants.

Key Words: *Agrobacterium tumefaciens,* β-glucuronidase (GUS), intron

Transgenik Tütün Bitkilerinde GUS Geni'nin Anlatımı ve Kalıtımı

Özet: *Agrobacterium tumefaciens* aracılığı ile tütün yaprak disklerine yapılan transformasyonda pBI 121 ve pGus-Int transformasyon vektörleri kullanılmıştır. Her iki vektörde de neomisin fosfotransferaz II (*npt II*) geni bulunmaktadır ve pGus-Int vektörü pBI 121'den farklı olarak β-glukuronidaz (GUS) geni içinde taşınabilir bir bitki intronu taşımaktadır. Moleküler işaret genlerinin döllerde varlığı birincil transformantların yanısıra, R1 ve R2 döllerinde de polimeraz zincir reaksiyonu (PCR) ve Southern blot analizleri ile doğrulanmıştır. GUS geninin aktivitesi ayrıca histokimyasal ve florometrik olarakda belirlenmiştir. Transformasyona kanıt olarak, kanamisin direnç özelliğine ait açılımlar döllerde gösterilmiştir. Analizler, transgenlerin sonraki döllere Mendel kurallarına uygun olarak aktarıldığını ve bazı transformantlarda intron dizilerin GUS gen aktivitesini %25-30 arttırdığını göstermiştir.

Anahtar Sözcükler: *Agrobacterium tumefaciens,* β-glukuronidaz (GUS), intron.

Introduction

Molecular manipulation is becoming very important in plant improvement. Recent advances in plant biotechnology provide a powerful tool for construction of genetically improved transgenic plants (1). Some chimeric gene constructs can be stably introduced into the plant genomes by different techniques such as electroporation, biolistics and protoplast fusion (2). In addition, *Agrobacterium*-mediated gene transfer system is widely utilized in the studies of plant molecular biology, especially for dicotyledonous. *Agrobacterium tumefaciens* is a gram-negative soil bacterium that infects wound sites of plant species and induces the development of crown-gall tumors (3). In this natural genetic engineering system, a specific region of Ti (Tumour-inducing) plasmid which is called transferred DNA (T-DNA) is transferred from *Agrobacterium* to a nuclear genome of the plant cells. The use of a reporter gene system simplifies the expression analysis of the gene in transgenic plants to follow its inheritance in the progeny. *E. coli*-originated β-glucuronidase is a widely used scorable marker gene and has been engineered for expression in a variety of organisms (4).

Normally, the GUS gene is absent in plant tissues and no detectable background was obtained in most higher plant cells. The sensitivity of assay systems make this marker useful for verification of transformation. The expression of GUS gene is detectable by fluorogenic and chromogenic subtrates. The histochemical GUS assay determines the tissue specific expression of the enzyme activity. X-gluc (5-bromo-4-choloro-3-indolyl-β-glucuronic acid) is a very efficient choromogenic substrate

for histochemical localization of β-glucuronidase activity in tissues and cells, giving a blue precipitate at the site of the enzyme activity. On the other hand, the fluorometric assay provides quantitation of GUS activity. Cleavage of the substrate 4-MUG (4-methyl umbelliferyl β-D-glucuronide) by GUS leads to the generation of a fluorogenic product 4-MU (4-methyl-umbelliferone), which is maximally fluorescent, in specific wavelengths.

The splicing mechanism is a nuclear process of many eukaryotic genes from which intervening sequences (IVSs) are removed pre-mRNA efficiently (5). The construct of the intron-GUS reporter gene system is useful for monitoring the transfer of the chimeric gene casette from bacteria into the plant cell in the early stages of transformation (6). Interruption of a reporter (indicator) gene by a plant intron prevents expression of the reporter gene at the bacterial level and allows the expression of gene only in plants (7).

The aim of this study was to observe the difference between the activities of intron-containing and intronless GUS genes in transgenic tobacco plants and the inheritance of the GUS gene in their generations.

Materials and Methods

Agrobacterium-mediated transformation

Agrobacterium tumefaciens strain LBA 4404 was used as a transformation vehicle to mediate gene transfer into tobacco (*Nicotiana tabacum* cv. L. Samsun). Plant expression vectors of pBI 121 and pGus-Int were mobilized into *A. tumefaciens* via triparental mating (8) with pRK 2013 as helper plasmid. The vectors have a selectable marker neomycin phosphotransferase (*npt II*), which confers resistance to kanamycin. The GUS and intron-containing GUS genes were under the control of CaMV 35S promoter and the transformation was carried out by leaf disc method as described by Horsch et al. (9). Leaf discs of tobacco 0.5 cm in diameter, infected with plasmid-harboring *A. tumefaciens* having final optical density as OD_{600}= 0.4. Primary transgenic discs (TR) were selected on 100 µg/ml kanamycin - and 500 µg/ml cefotaxime-containing Murashige - Skoog (MS) plates (10).

PCR and Southern analyses of putative transgenics

Genomic DNA was isolated from 2-month-old plant leaves according to Edwards et al. (11). PCR was run with specific forward and reverse primers (GUS 1 5' GGT GGG AAA GCG CGT TAC AAG 3' and GUS 2 5' GTT TAC GCG TTG CTT CCG CCA 3') of the GUS gene and 35 cycles were driven by thermocycler for 60 sec at 95°C, 60 sec at 36°C and 90 sec at 72°C. The PCR was were completed for 10 min at 72°C.

Southern hybridization was performed with *Xba*I digested genomic DNAs (8 µg) to determine the integration of the GUS gene into plant genomes and to estimate the number of insertion in the transformants. The overnight digested DNA was fractionated on a 1% agarose gel and transferred to nylon membrane with fixation by UV crosslinking. The non-radioactive digoxigenin (DIG) hybridization system was used for hybridization analysis. The PCR-amplified product of the GUS gene was labelled with DIG-dUTP and used as a probe for Southern hybridization. Hybridization was carried at 68°C and immunological detection steps were performed according to manufacturer's instructions (DIG-DNA Labelling and Detection Kit of Boehringer Mannheim, Germany).

Histochemical and fluorometric GUS assays

The histochemical GUS assay was also performed with the leaves of 2-month-old plantlets. They were washed for 30 min with 50 mM phosphate buffer (pH 7.0) and immersed for 10 min in fixation solution (0.3% formaldehyde, 10 mM MES, 0.3 M mannitol). The samples were put in 1mM X-gluc (5-bromo-4-choloro-3-indolyl-β-glucuronic acid) solution and incubated at 37°C overnight for blue color development. GUS activity was also detected with overnight cultures of *Agrobacterium* harboring pBI 121 and pGus-Int by histochemical assays. The proteins were extracted from the leaves (12) and the amount of protein was determined according to the Bradford assay with bovine serum albumin as standard (13).

Quantitative determination of GUS activity was accomplished by fluorometric GUS assay. Protein extraction of 10 µg was incubated with 1 mM MUG buffer at 37°C for 90 min according to Jefferson et al. (12). The enzymatic reaction was measured by spectrofluorometer (Photon Technology International) with excitation at 365 nm and emission at 455 nm. The fluorometer was calibrated with a fresh preparation of MU (100 nm) as standard. Expression assays of the GUS gene were also carried out with the stem and root section of regenerants in addition to leaf sections. The relative fluorescence readings of the samples were obtained as curve, and data from the curve were applied to the formula to obtain a diagram of GUS activity. The quantitative measurements of GUS activity were expressed as 'pmol metylumbelliferone (MU)/mg protein/min' units.

Progeny tests

R1 and R2 plants (first and second generations) are seed-derived plants obtained from the self-pollination of primary transformants (TR) and R1 plants, respectively. Seeds of R1 and R2 plants were surface-sterilized and were germinated on 100 µg/ml kanamycin containing MS medium. Five-week-old green plantlets with several leaves were scored as resistant (R), and white or non-germinated ones were scored as sensitive (S). Statistical analyses were performed by the counting R_1 and R_2 seeds on kanamycin-containing selective MS media. Distribution of progeny was tested against the expected ratios using the chi-square (χ^2) test, which was used to analyze segregation of the *npt II* gene in the progenies.

Results

Following the transformation of the leaf discs, putative transformants were selected based on regeneration under kanamycin-containing MS plates, and transformation efficiency was observed 90%. There were no morphological differences between control and transgenic plants; however, flower formation occured two months later in transgenic plants than in control plants. The genomic DNA from the leaves of 2-month-old putative transgenic plants were extracted and amplified with specific primers for the GUS gene. PCR products of 1.4 kb and 1.2 kb fragments were obtained from the DNA of plants to which intron-containing and intronless GUS genes had been transferred, respectively (Figure 1). A difference of approximately 200 bp was obtained from the intron located in the GUS gene. In addition to the PCR of the GUS gene, the *npt II* gene was also amplified and 0.8 kb bands were obtained with transgenic plant genomes (data not shown).

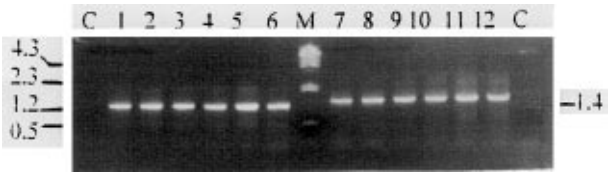

Figure 1. PCR analysis of transgenic tobacco plants tranformed with pBI 121 (1-6) and pGus-Int (7-13). C, control plant. *Hin*d III digested lambda DNA was used as size marker (M).

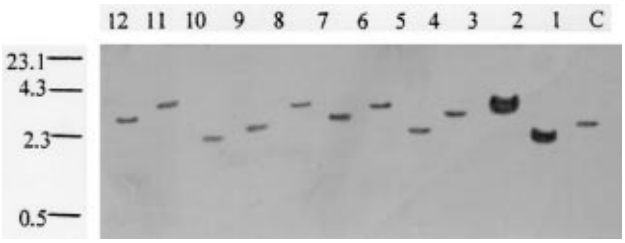

Figure 2. Southern blot analysis of R1 progenies of tobacco plants transformed with pGus-Int (R1GI; 1-6) and pBI 121 (R1G; 7-12). C, control plant.

The data show that the chimeric DNAs were stably transmitted to further generations without alterations in gene integration. Southern analysis indicated that the tested transgenic plants contained from 1-to-2 inserts of the GUS gene in their genomes (Figure 2).

Histochemical GUS assay was performed by incubation of putatively transgenic tobacco leaves with X-gluc overnight, blue color formation occured in the expression sites where X-gluc was catabolized by GUS gene product (data not shown). Incubation of the overnight culture of *Agrobacterium* having pBI 121 with X-gluc exhibited GUS activity by changing the color of the culture to blue. As expected, the intron-containing GUS reporter gene construct showed no GUS activity in pGus-Int-containing cultures of *Agrobacterium* where the intron-splicing process normally does not occur. However, GUS expression was visualized histochemically in *Agrobacterium* cultures having the pBI 121 plasmid.

Quantitative GUS activity was determined by measurement of fluorescence produced by conversion of MUG to MU by GUS gene product (Figure 3). Fluorometric analysis provides quantitative determination of the activity of the GUS gene when the leaf homogenate is incubated with MUG. The curves were obtained by fluorometer and peak points were used to calculate GUS activity. The activity diagram shows that the expression of intron containing GUS transformants were 25-30% higher than intronless GUS containing transformants. We did not obtain any significant GUS activity in control tobacco plants.

Segregation analyses were performed to characterize

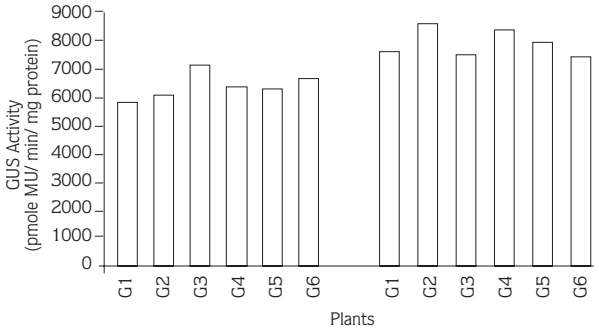

Figure 1. PCR analysis of transgenic tobacco plants tranformed with pBI 121 (1-6) and pGus-Int (7-13). C, control plant. *Hin*d III digested lambda DNA was used as size marker (M).

Table 1. Segregation of the *npt II* gene in R1 and R2 progeny plants of TR_G line transformed with pBI 121 plasmid.

Generation	Kan^R	Kan^S	χ^2
R1G-1	76	24	7.68
R1G-2	82	19	8.91
R1G-3	74	25	4.55
R1G-4	70	20	3.81
R1G-5	73	24	3.80
R1G-6	80	22	3.90
R1G-7	78	24	3.57
R2G-1	71	25	4.55
R2G-2	81	20	5.35
R2G-3	73	23	3.00
R2G-4	72	23	2.22
R2G-5	72	25	5.50
R2G-6	76	22	5.07
R2G-7	75	20	9.88

Table 2. Segregation of the *npt II* gene in R1 and R2 progeny plants of TR_{GI} transformed with pGus-Int plasmid.

Generation	Kan^R	Kan^S	χ^2
R1GI-1	79	20	7.68
R1GI-2	80	19	8.91
R1GI-3	76	23	4.55
R1GI-4	78	25	3.81
R1GI-5	73	23	3.80
R1GI-6	76	24	3.90
R1GI-7	77	25	3.57
R2GI-1	76	23	4.55
R2GI-2	76	21	5.35
R2GI-3	72	24	3.00
R2GI-4	73	26	2.22
R2GI-5	77	22	5.50
R2GI-6	69	21	3.81
R2GI-7	80	18	9.88

Kan^R and Kan^S, kanamycin-resistant and kanamycin-sensitive seedlings.

transforming DNA for the *npt II* gene in R1 and R2 generations. The primary regenerants obtained from transformed leaf discs were rooted and the plants exhibiting GUS expression in their leaves were selfed for seed production. R1 and R2 seeds derived from self-pollination of the primary regenerants (TR_G and TR_{GI}), harvested and allowed to germinate on kanamycin-containing MS medium (Table 1,2). χ^2 analysis indicated a 3:1 segregation model for the *npt II*, a ratio that correlates with Mendelian segregation of a single dominant gene. Kanamycin-resistant plants were also confirmed for GUS gene activity and results indicated that the *npt II* and the GUS gene activities show correlation in F1 and F2 generations.

Discussion

The *Agrobacterium*-mediated binary vector system is an excellent model of the natural exchange of genetic material from a prokaryote to an eukaryote. In this study, *Agrobacterium tumefaciens* was used for the delivery of foreign DNA into the tobacco genome. The characterization of the marker gene integration into putative transgenic plants and its inheritance in R1 and R2 plants were analyzed. Transgenic seeds of primary transformants were randomly selected on the basis of kanamycin resistance. The frequency of resistant and sensitive seedlings were also determined for information concerning the segregation of the transferred genes.

The PCR analyses showed that both the GUS and the *npt II* genes were transmitted to the progenies and were co-inherited.

Southern blot analysis revealed that most of the primary transformants contained one copy of the transgene. Most of the progenies also had one copy of the transgene, but some of the progenies of R2 (Figure 4, lanes 2 and 3) had more than one copy of the transgene in their genomes. Southern analysis is concordant with the enzyme assays, with regenerants exhibiting enzyme activity having foreign DNA in their genomes. Seedlings from self-pollinated plants segregated 3:1 kanamycin resistant: sensitive, indicating that insertions appeared as low copies in the Southern analyses of progenies.

Histochemical analysis of the GUS gene showed that the staining intensity of GUS expression was stronger in the tissues of the intron-GUS plants than in the intronless

GUS plants. This is also supported by fluorometric assays where GUS activity was measured quantitatively. The expression of the GUS gene is slightly higher in plants transformed with the intron containing GUS gene than in plants transformed with the intronless GUS gene.

χ^2 is used to determine the correlation between theoretical and experimental (in selective media) ratios of the progenies according to Mendelian segregation. Probabilities were greater than 0.05 and the results indicated that the transgenic tobacco plants produced by *Agrobacterium*-mediated transformation method were genotypically and phenotypically stable in the progenies. The values obtained from experiments correlate with the 3:1 Mendelian ratio. The 3:1 segregation ratio for kanamycin resistance would also come from a single copy of the *npt II* gene. In most cases, the transformed genes behave as a single dominant locus exhibiting normal Mendelian segregation. In previous studies on transgenic plants, Mendelian and non-Mendelian segregation of transgenes have been observed (14, 15). It is reported that single T-DNA insertion results in high levels of transgene expression; however, multiple copies of transgenes lead to supression of the chimeric gene in some cases (16). Stability and integration of the transgenes to progeny plants were investigated with genomic DNA isolated from transgenic plants.

Expression studies of the GUS gene activity were carried out with different organs of transgenic plants, namely the leaf, stem and root. We obtained similar results in the activities of different organs and we concluded that splicing of the intron was not different in various organs of tobacco in terms of expression of the constitutive 35S promoter.

It has also been reported that the location of the intron within the fusion gene is important (17) and stimulation of gene expression by intron insertion is closely dependent on the location of the intron in maize cells (18). It is also claimed that insertion of an intron near the 5'end of mRNA yields more profound effects. In our pGus-Int vector the intron from the potato was located at a codon for 128th amino acid residue from the N-terminus of the GUS polypeptide (17). In our heterologous system, transformation of the potato intron into tobacco plants increased expression of the GUS gene by 25-30%. Inclusion of introns in the transcription unit seems to influence the stability of the transcript, leading to an enhancement of gene expression of up to 71 and 26 fold in maize and bluegrass, respectively (7).

A very efficient way to prevent expression of the reporter gene in bacterial level is to place an intron inside the gene to allow its reliable expression only in plants. In our transgenic tobacco plants, the chimeric GUS-Intron gene is spliced out efficiently, thus giving a functional and highly expressed mRNA.

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