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Expression and molecular analysis of *DsRed* and *gfp* fluorescent genes in tobacco (*Nicotiana tabacum* L.)

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ABSTRACT

Agrobacterium-mediated transformation of tobacco leaf disks with *Agrobacterium tumefaciens* (*A. t.*) strain LBA4404 and two plasmids (pCAMBIA1390-DsRed and pART27 2mgfp5-ER) was used for introducing red fluorescent gene (*DsRed*), green fluorescent gene (*gfp*) and corresponding selection genes (*hptII* for resistance to antibiotic hygromycin and *nptII* for resistance to kanamycin) into leaf discs of tobacco (*Nicotiana tabacum* L.). Epifluorescent microscopy with the appropriate set of filters did not reveal phenotypic expression of the *DsRed* gene in 6.9 % of regenerants and the *gfp* gene in 1.3 % of regenerants that were successfully grown on selective medium. The duplex PCR method also did not confirm the presence of fragments specific to *DsRed* or *gfp* genes in these regenerants, while the presence of fragments characteristic of selection genes *hptII* and *nptII* was confirmed. A built-in *nptII* gene mutation, a deletion, was detected in one regenerant. Out of the 139 regenerants generated after the transformation of *A. t.*-pCAMBIA1390-DsRed, 38 or 25.5 % successfully grew only on non-selective medium; after transformation with *A. t.*-pART27 2mgfp5-ER 9 or 5.4 % of the 161 generants grew successfully. PCR analysis confirmed in all regenerants the presence of fragments characteristic of both transgenes, which were not expressed or were silenced. The effectiveness of transformation after infection with *A. t.*-pCAMBIA1390-DsRed was 93.1 %, and 98.7 % after infection with *A. t.*-pART27 2mgfp5-ER. We established that both fluorescent genes are suitable for setting up a transformation system. The antibiotics hygromycin and kanamycin successfully prevented the growth of untransformed tissues, but the antibiotic timentin successfully prevented the growth of bacteria *A. t.* after the transformation.

Key words: *Nicotiana tabacum*, fluorescent genes, selection genes, transformation, exspression of transgenes, DNA analysis

IZVLEČEK

IZRAŽANJE IN MOLEKULSKA ANALIZA *DsRed* IN *gfp* FLUORESCENTNIH GENOV PRI TOBAKU (*Nicotiana tabacum* L.)

Z metodo posredne transformacije z vektorskim sistemom *Agrobacterim tumefaciens* (*A. t.*) sev LBA4404 in dvema plazmidoma (pCAMBIA1390-DsRed in pART27 2mgfp5-ER) smo v listne izsečke tobaka (*Nicotiana tabacum* L.) vnesli fluorescentni markerski gen za rdečo (*DsRed*) oz. zeleno (*gfp*) fluorescenco ter selekcijska gena za odpornost na antibiotik higromicin (*hptII*) oz. kanamicin (*nptII*). Z epifluorescentnim mikroskopom in ustreznim setom filtrov nismo zasledili fenotipskega izražanje *DsRed* gena pri 6,9 % regenerantih in *gfp* gena pri 1,3 % regenerantih, ki so uspešno rastli na selekcijskem gojišču. Pri teh regenerantih tudi z dupleks PCR metodo nismo potrdili prisotnosti fragmentov značilnih za *DsRed* oz. *gfp* gen, medtem ko smo potrdili prisotnost fragmentov značilnih za selekcijska gena *hptII* in *nptII*. Pri enem regenerantu smo v vgrajenem *nptII* genu zasledili mutacijo in sicer delecijo. Od 139 nastalih regenerantov, po transformaciji z *A. t.*-pCAMBIA1390-DsRed, jih je 38 oz. 25,5 % uspešno rastlo le na neselekcijskem gojišču, po transformaciji z *A. t.*-pART27 2mgfp5-ER je bilo takih 9 oz. 5,4 % od 161 nastalih. Pri vseh smo s PCR analizo potrdili prisotnost fragmentov značilnih za oba transgena, ki se nista izražala oz. sta bila utišana. Učinkovitost transformacije po okužbi z *A. t.*-pCAMBIA1390-DsRed je bila 93,1 %, po okužbi z *A. t.*-pART27 2mgfp5-ER pa 98,7 %. Ugotovili smo, da sta oba fluorescentna gena primerna za vzpostavitev transformacijskega sistema. Antibiotika higromicin in kanamicin sta uspešno preprečila rast netransformiranih tkiv, antibiotik timentin pa je uspešno preprečil rast bakterije *A. t.* po transformaciji.

Ključne besede: *Nicotiana tabacum*, fluorescentni geni, selekcijski geni, transformacija, izražanje transgenov, DNA analiza

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1 INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) has been shown to be a very suitable model plant for genetic transformation because it grows quickly and successfully in tissue culture. Regeneration from leaf explants is fast and efficient (Stolarz *et al*., 1991). Tobacco was the first transformed plant. In 1983, the gene for resistance to the antibiotic kanamycin was inserted in tobacco (Horsch *et al*., 1984) by the indirect method of transformation using the soil phytopathogenic bacterium *Agrobacterium tumefaciens* (*A. t.*). There are currently two authorizations for commercial production of tobacco with tolerance to the herbicide oxynil in the European Union and for tobacco with a reduced content of nicotine in the United States of America (CERA, 2012).

The development of plant regeneration procedures and the discovery of new techniques of gene transfer in plant cells have provided opportunities for practical application of genetic engineering to modify and improve important agricultural crops. Genetic transformation has become useful in improving plant properties and for the detection of gene functions in plants (Rao *et al*., 2009).

In most cases, only a small proportion of plant cells transform, so it is necessary to enter a selection gene together with the desired gene, by which transformed cells can be distinguished from non-transformed ones. Selection can be positive or negative (Miki and McHugh, 2004). The correct concentration of antibiotic or selection agent must be made, which completely prevents regeneration of non-transformed cells and, at the same time, minimizes the number of non-transformed regenerants that develop in cultured explants due to the detoxification activity of the surrounding transformed cells (Park *et al*., 1998).

Using selection genes for antibiotic resistance and resistance to herbicides gives rise to most concerns in the commercial use of transgenic plants. DNA transfer between transgenic plants and other organisms is unlikely. *NptII* gene does not signify any risk to human and animal health (Fuchs *et al*., 1993). Nevertheless, the complete removal of the selection gene is desirable because selection genes are no longer required after selection. It would probably also contribute to the greater acceptance of genetically modified plants. There are quite a few successful methods of removing these genes (Afolabi, 2007). European regulations governing the release of genetically modified plants in the field prohibit the inclusion of genes for resistance to antibiotics.

Test or marker genes are genes whose gene product can be visually identified and their location determined. They enable the quick identification of transformed tissues. Marker genes that can be detected through other senses, such as taste or smell, can also be useful (Witty, 1989).

Genes for the synthesis of fluorescent proteins have advantages over other marker genes because they can be visually detected in living cells without the use of invasive procedures using substrates and products that could diffuse within or between cells. Transformed cells, in which these genes express, can be identified shortly after the transformation and it can be determined whether they are dividing (Harper *et al*., 1999). Fluorescent proteins in fusion with any proteins allow monitoring of the location, movement and activities of proteins in living cells. They can be used as markers for tracing and tracking proteins, discovering interactions between proteins and tracking the destiny of proteins in the cell (Lippincott-Schwartz and Patterson, 2003). Fluorescent proteins can also be used to monitor the destiny of transgenes introduced into cultivated plants and their impact on the environment (Stewart, 2005).

The best known fluorescent protein is the green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*) (Haseloff and Amos, 1995), which emits green fluorescence under illumination with long-wave UV light. The wild-type *gfp* gene was modified in such a way that it effectively reflects in plants and the spectral properties and fluorescence were changed and improved (Reichel *et al*.,1996; Haseloff *et al*., 1997).

Red fluorescent protein DsRED was isolated from coral (*Discosoma* sp.) and, using appropriate filters, can be more easily separated from autofluorescent chlorophyll (Matz *et al*., 1999) than GFP. *DsRed* gene is used as a marker gene for transient and stable transformation of tobacco and,

in combination with the *gfp* gene, is suitable for simultaneous monitoring of the expression of the two genes (Jach *et al*., 2001).

Many fluorescent proteins that are useful for studies of genetic transformations have been discovered. Orange fluorescent proteins have proved to be very successful as marker genes, especially TdTomato-ER, which fluoresces the brightest of all fluorescent proteins, followed by Morang-ER (Mann *et al*., 2012).

In this study, we monitored the phenotypic expression of *DsRed* and *gfp* fluorescent genes and selection genes *hpII* and *nptII*, as well as molecular analysis of their insertion into the genome of tobacco.

2 MATERIALS AND METHODS

2.1 Plant material

The leaves of micropropagated tobacco variety Havana 38 were used for transformation.

2.2 Bacteria and plasmids

The commercial bacterium *A. t*. strain LBA4404 was chosen for gene insertion, in which modified plasmid pCAMBIA1390-DsRed (Cambia, 1997; Škof, 2008) or plasmid pART27 2mgfp5-ER was introduced by electroporation (Gleave, 1992).

Plasmid pDsRed-Express contains the gene *DsRed-Express*, which is a form of red fluorescent protein DsRED. For preparation of the plasmid vector with the *DsRed* marker gene, the gene for DsRED-Express protein from plasmid pDsRed-Express (BD Bioscience Clontech, Palo Alto, USA) was used, which was equipped with a constitutive CaMV35S promoter from the vector pBIN m-gfp5-ER and included in the plasmid vector pCAMBIA1390 (Cambia, Canberra, Australia) (Škof, 2008). In addition to the *DsRed* marker gene, the plasmid contained the plant selection *hptII* gene for resistance to the antibiotic hygromycin for selection of transformed plant tissues and the *nptII* selection gene for resistance to the antibiotic kanamycin for selection of transformed bacteria (Table 1).

Plasmid pART27 2mgfp5-ER is a binary vector, which was prepared in the laboratory of Prof. Dr. C. C. Eady (Institute of Crop and Food Research, Christchurch, New Zealand), in such a way that two repetitions of mgfp5-ER gene from the vector pBIN m-gfp5-ER were included in the plasmid vector pART27 at location *Spe*I of the multiple cloning site (MCS). pART27 vector contains the selection gene *spec* for resistance to the antibiotic spectinomycin for selection of transformed bacteria and the *nptII* gene for resistance to the amino glycoside antibiotics geneticin and kanamycin for selection of transformed plant tissues (Table 1) (Gleave, 1992).

Table 1. Plasmids with bacterial and plant selection and fluorescent genes

Plasmid	Bacterial selection	Gene	Plant selection	Gene	Fluorescent protein	Gene
pCAMBIA1390-DsRed	kanamycin	*nptII*	hygromycin	*hptII*	DsRED	*DsRed*
pART27 2mgfp5-ER	spectinomycin	*spec*	kanamycin, geneticin	*nptII*	GFP	*gfp*

2.3 Agrobacterium-mediated transformation

Transformation of tobacco with *A. t*. was performed using a slightly modified method of transformation of leaves after Horsch *et al*. (1985) and Fisher and Guiltinan (1995). Tobacco leaves were cut under sterile conditions to explants of about 1 cm^2. For plasmid pCAMBIA1390-DsRed 105 leaf explants were prepared and for plasmid pART27 2mgfp5-ER 103 explants.

Bacterial suspensions of *A. t*., with the appropriate plasmid included, were incubated overnight at 28 °C and shaken at 120 rev./min. in *YEB* medium

[sucrose 5 g/l, peptone 5 g/l, beef extract 5 g/l, yeast extract 1 g/l, $MgSO_4 \times 7H_2O$ 1 g/l; pH 7.0]. Bacterial suspensions were centrifuged at 5000 rpm for 5 min. The supernatant was removed and the *Agrobacterium* pellet was resuspended in ½*MS* liquid basal medium (Murashige and Skoog, 1962) at an optical density of OD_{600nm} = 0.5 (5×10^6 cells/ml). Tobacco leaf explants were incubated in Petri dishes for approximately 20 min in the *A. t.* suspension with the appropriate plasmid and then gently dried on sterile filter paper in a laminar flow cabinet and co-cultivated on *MSr* medium with the addition of [Fe-Na_2-EDTA 0.1 mg/l, thiamine 0.1 mg/l, BAP 1.0 mg/l, NAA 0.1 mg/l, acetosyringone 100 µM, agar 8 g/l; pH 5.8] (Stolarz et al., 1991). After three days of co-cultivation, they were washed twice in a solution of antibiotic timentin 200 mg/l [100:1 (w/w) ticarcillin: clavulanic acid] and air-dried.

Then, the leaf explants were transferred onto selective *MSr* medium without acetosyringone and with the addition of timentin 150 mg/l to prevent the growth of *A. t.* bacteria and an appropriate selection antibiotic (Table 1). The minimum effective concentration of selection antibiotics was chosen, i.e., 25 mg/l hygromycin antibiotic for the selection of tobacco transformants after infection with *A. t.*-pCAMBIA1390-DsRed and 300 mg/l of the antibiotic kanamycin after infection with *A. t.*-pART27 2mgfp5-ER. Explants were cultured in a growth chamber at a 16/8 hour photoperiod and a temperature of 24 ± 1 °C, illuminated with about 40 µmol/m^2s. After five weeks, the explants were transferred or sub-cultured on the appropriate fresh selective *MSr* medium. The resulting regenerants were transferred onto *MSm* medium with the addition of the appropriate selection antibiotic, without timentin. After five weeks, the regenerants that had successfully grown were transferred to the appropriate *MS* selective medium. Regenerants that had grown poorly or had begun to decay were transferred to *MSm* medium without selection antibiotics in order to determine the presence of the selection transgene and its expression.

2.4 Expression of *DsRed* and *gfp* genes

Expression of fluorescent marker genes in the regenerants was observed after infection at the beginning of regeneration in the rising stages of pessaries or inception. Transformed tobacco explants were examined by epifluorescent microscope (Nikkon SMZ 1000) at 20× magnification and appropriate filters for the detection of the red fluorescence *DsRed* gene and the green fluorescence *gfp* gene. For the detection of red fluorescent protein DsRED-Express (plasmid pCAMBIA1390-DsRed), which has an excitational maximum at 557 nm and emission maximum at 579 nm, a set of filters with EX 546/10 nm, DM 575 nm and BA 620 nm was used. For the detection of green fluorescent protein m-GFP5-ER (plasmid pART27 2mgfp5-ER), which has an excitational maximum at 484 nm and emission maximum at 510 nm, a set of filters with EX 480/40 nm, DM 505 nm and BA 535/50 nm was used.

2.5 Molecular analysis of plant material by PCR method

For DNA analysis of the presence of transgenes in tobacco regenerants, the complete DNA was isolated, the overall concentration of isolated DNA was measured, dilutions to 20 ng/µl were prepared, and polymerase chain reaction (PCR) and fragment analysis amplified with agarose gel electrophoresis were performed.

Isolation of DNA from plant tissue

Overall genomic DNA from the leaves of non-transformed tobacco were isolated - negative control and transformed regenerants, as well regenerants that had only prospered on non-selective mediums without antibiotics, according to the method of Kump *et al*. (1992).

Measuring the concentration of DNA by fluorimeter

The concentration of isolated DNA in solution was measured using a DNA fluorimeter DyNA Quant™ 200 (GE Healthcare). A working solution was prepared from 10×TNE buffer [0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 7] and colorant Hoechts 33258 added in a final concentration of 0.1 µg/ml. Calf thymus DNA (1 mg/ml DNA in 1×TNE buffer) was used for calibration of the fluorimeter. For each sample of DNA, 2 ml of the working solution and 2 µl DNA sample were added to the cuvette, the mixture stirred and the concentration of DNA then measured. DNA samples were diluted to 20 ng/µl.

Polymerase chain reaction (PCR)

Specific multiplication of *DsRed* and *gfp* genes was carried out in duplex PCR reactions using two pairs of primers (Table 2). For analysis of the inclusion of *DsRed* and *hptII* genes in the plant genome after transformation with *A. t.* and the plasmid pCAMBIA1390-DsRed, a combination of REDfor/RED2right and HPTII-for/HPTII-rev1 primers was used. A combination of GFP1a/GFP1b and NPTII1a/NPTII1b primers was used for analysis of the inclusion of mgfp5-ER and *nptII* genes after transformation with *A. t.* and the plasmid pART27 2mgfp5-ER.

PCR reaction mixtures were prepared in a laminar flow cabinet. A 5 µl DNA sample was pipetted into the PCR microfuge. Samples were centrifuged at 1000 rpm/min and 1×PCR buffer [10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40] (Fermentas), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), 4×0.5 µM suitable primer and 0.5 units of enzyme Taq DNA polymerase (Fermentas) were added. The final volume of the reaction mixture in which multiplication of DNA was conducted, was 25 µl. The PCR reaction was carried out in a cyclical thermostat GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the modified temperature model (Lakshmi *et al*., 1998):

- initial denaturation of 5 min at 94 °C,
- 33 repeated cycles: - denaturation of DNA 1 min at 94 °C,

- annealing of primers 1 min at 58 °C,
- synthesis of DNA fragments 1.5 min at 72 °C,

- final incubation 7 min at 72 °C.

Samples were stored at 12 °C until further analysis.

Table 2: DNA nucleotide sequences of primers for an individual transgene and the expected length of the amplified fragment

Primer	The nucleotide sequence 5' - 3'	Expected length of the fragment (bp)
GFP1a	AGT GGA GAG GGT GAA GGT GAT G	422
GFP1b	TTG TGG CGG GTC TTG AAG TTG G	
REDfor	AGG ACG TCA TCA AGG AGT TCA T	211
RED2right	GTG CTT CAC GTA CAC CTT GGA G	
HPTII-for	ATG ACC GCT GTT ATG CGG CCA TTG	641
HPTII-rev1	AAA AAG CCT GAA CTC ACC GCG ACG	
NPTII1a	GAG GCT ATT CGG CTA TGA CTG	650
NPTII1b	ATG GGG AGC GGC GAT ACC GTA	

Analysis of DNA fragments by agarose gel electrophoresis

For the separation of DNA fragments, horizontal electrophoresis was used on a 1.4 % gel [1.4 % SeaKem LE agarose (Cambrex, USA), 1×TBE buffer, Ethidium bromide 0.5 µg/ml], which was installed in an electrophoretic tank (Bio-Rad Sub-Cell, model 192) immersed in 1×TBE buffer [890 mM Tris, 890 mM boric acid, 10 mM EDTA]. Five µl dispensing dye BPB [12.5% (w/v) ficol 400, 0.2% (w/v) bromophenol blue, 6.7% (v/v) 10×TBE] were added to the samples, which were stirred and 17 µl of sample was applied on the agarose gel. In addition to the samples, on the gel were also applied: DNA isolated from control (non-transformed tobacco), corresponding pure plasmid (isolated from *E. coli*), a blind sample (all components of the reaction mixture except the DNA; instead of adding 5 µl of water) and a size standard (GeneRulerTM 100 bp DNA Ladder Plus (Fermentas) with 14 fragments: 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp). Electrophoresis was carried out at 140 V in the anode direction for about 1 hour and 30 min. The gel contained 0.5 µg/ml ethidium bromide, which, in a complex with double stranded DNA molecules, allowed their detection under UV light (302 nm). Gels were observed using a transilluminator TMF-30 (UVP Inc., UK) and photographed with a digital camera.

3 RESULTS AND DISCUSSION

3.1 Regeneration of tobacco leaf explants and phenotypic transgene expression

Leaf explants, after incubation with *A. t.* and an appropriate plasmid, were co-cultivated on *MSr* medium with added acetosyringone 100 µM, in order to increase the infection, as described by Sunilkumar *et al*. (1999). In nature, phenolic substances such as acetosyringone, which are released on wounding of plant tissue, trigger the activation of genes for virulence (*vir* genes) in infection with *Agrobacterium* (Gelvin, 2003). We obtained a high percentage of transformed regenerants, which can be attributed to the acetosyringone attached to the *MSr* medium in the period of co-cultivation. After the completion of co-cultivation, timentin 150 mg/l was added to the *MSr* medium, which effectively inhibited the growth of the *A. t*. bacteria but did not adversely affect regeneration. The regenerants on the medium with timentin were distinctly dark green. Nauerby *et al*. (1996) reported that timentin in this concentration completely prevented the multiplication of *A. t*. and positively impacted on the regeneration of leaf and cotyledon explants of tobacco. Similarly, Cheng *et al*. (1998) emphasized that timentin is just as effective as carbenicillin and cefotaxime and does not have an inhibitory effect on the regeneration of shoots in tobacco and Siberian elm.

Germs of the first regenerants occurred after 10-12 days, regardless of the built-in genetic construct. Regeneration was mostly direct, without an intermediate callus, as noted by Stolarz *et al*. (1991).

After five weeks, a large number of regenerants was observed. Regenerants from the leaf explants, in which phenotypic expression of the inserted fluorescent genes was observed, were transferred onto *MSm* medium with the addition of an appropriate selection antibiotic. After five weeks, regenerants that had grown poorly were transferred to *MSm* medium without added antibiotics, other regenerants were transferred to appropriate fresh *MSm* selective medium. The percentage of surviving and failed regenerants is given in Table 3.

Table 3: Percentage of surviving and failed regenerants of tobacco in the appropriate selective or non-selective *MSm* media after transformation with *A. t.* and plasmid pCAMBIA1390-DsRed or plasmid pART272mgfp5-ER

Plasmid	Percentage of regenerants on the medium				
	selective		non-selective		
	survived	failed	transferred	survived	failed
pCAMBIA1390-DsRed	67.8	6.7	25.5	25.5	0
pART27 2mgfp5-ER	90.4	1.2	8.4	5.4	3.0

After transformation with *A. t*.-pCAMBIA1390-DsRed, 149 regenerants were obtained from 105 explants. After sub-cultivation on selective *MSm* medium with 25 mg/l hygromycin, 101 or 67.8 % of regenerants grew successfully, and 10 or 6.7 % failed. On non-selective medium, all 38 regenerants (25.5 % out of 149), were successfully grown. With *A. t*. pART272mgfp5-ER, 103 tobacco explants were transformed and 168 regenerants were obtained. On the selective *MSm* medium containing 300 mg/l kanamycin, 152 or 90.4 % of regenerants successfully grew, and 2 or 1.2 % failed. Fourteen or 8.4 % of regenerants that had grown poorly or had begun to deteriorate on the selective medium, were transferred to non-selective medium. Out of them, 9 or 5.4 % grew successfully, while 5 or 3 % failed (Tables 3 and 4).

With 6.9 % of regenerants examined by epifluorescent microscope, no red fluorescence DsRED protein was observed and with 1.3 % of

regenerants no green fluorescence m-GFP5-ER protein was detected, despite the fact that they had successfully grown on the selection media (Tables 4 and 5). There had been non-expression or silencing of the fluorescent genes.

3.2 Molecular analysis of transgene integration

DNA analysis was performed on all 300 surviving regenerants, whether or not they had been transferred to non-selective *MSm* medium (Tables 4 and 5).

Table 4: Number and percentage of regenerants and transgenes of tobacco after transformation with *A. t.* - pCAMBIA1390-DsRed on selective and non-selective *MSm* medium.

Number of regenerants on *MSm* media	Number or percentage of transgenes					
	DsRed in *hptII*		*DsRed*		*hptII*	
	number	percentage	number	percentage	number	percentage
101 on selective	94	93.1	0	0.0	7	6.9
38 on non-selective	38	100	0	0.0	0	0.0
139 together	132	95.0	0	0.0	7	5.0

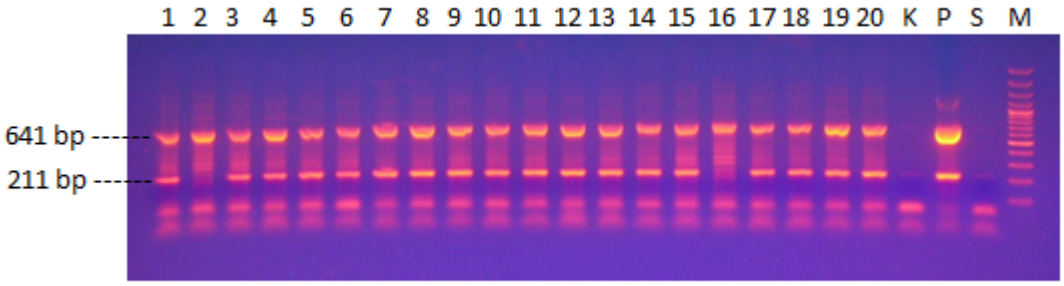

Figure 1: Multiplied DNA fragments in duplex PCR reaction with the pair of primers for the *DsRed* gene (211 bp) and the pair of primers for the *hptII* gene (641 bp). The figure shows only the first 20 regenerants of 139. (1-101 - transformed regenerants of tobacco grown on selective medium, 102-139 - transformed regenerants of tobacco grown on non-selective medium), K - control - non-transformed tobacco, P - plasmid pCAMBIA1390-DsRed, S - blind sample M - size standard

In all regenerants of tobacco transformed with *A. t.*-pCAMBIA1390-DsRed that were grown on selective medium (regenerants 1-101) and regenerants that, due to poor growth or decay were transferred to non-selective medium (regenerants 102-139), the presence of fragment length 641 bp (selection *hptII* gen) was detected. In regenerants 2, 16, 49, 51, 53, 61 and 78, only the presence of fragment length 641 bp was detected, but not the presence of fragment length 211 bp, which would have confirmed the presence of marker *DsRed* gene. On the selective medium, 93.1 % of regenerants included both transgenes from the genetic construct, thus both *DsRed* and *hptII,* and 6.9 % regenerants only part of the genetic construct, with the *hptII* gene (Figure 1 - only 20 out of 139 regenerants are presented, Table 4).

For all 38, or 25.5% of regenerants that were transferred to the non-selective medium, molecular analysis determined the presence of both transgenes from the gene construct (Table 4). Epifluorescent microscopy revealed the presence of protein DsRED in all regenerants, at least mosaic expression. Despite the confirmed presence of selection gene *hptII*, which should disintegrate the hygromycin added to the medium and allow normal growth and development of regenerants, they decayed. This suggests that gene *hptII* phenotypically did not express or was silenced (Figure 1 - only 20 out of 139 regenerants are presented, Tables 3 and 4).

Table 5: Number and percentage of regenerants and transgenes of tobacco after transformation with *A. t.*-pART27 2mgfp5-ER on selective and non-selective *MSm* medium

Number of regenerants on *MSm* medium	Number or percentage of transgenes					
	gfp in *nptII*		*gfp*		*nptII*	
	number	percentage	number	percentage	number	percentage
152 on selective	150	98.7	0	0.0	2	1.3
9 on non-selective	9	100	0	0.0	0	0.0
161 together	159	98.8	0	0.0	2	1.,2

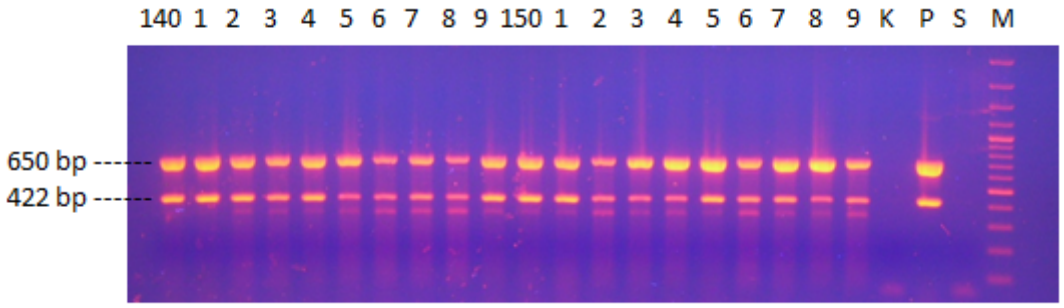

Figure 2: Multiplied DNA fragments in duplex PCR reaction with the pair of primers for the *gfp* gene (422 bp) and the pair of primers for the *nptII* gene (650 bp). The figure shows only the first 20 regenerants of 161. (140-291 - transformed regenerants of tobacco grown on the selective medium, 292-300 - transformed regenerants of tobacco grown on the non-selective medium), K - control - non-transformed tobacco, P - plasmid pART27 2mgfp5-ER, S - blind sample M - size standard.

In the regenerants of tobacco transformed with *A. t.*-pART27 2mgfp5-ER, which were grown on the selective medium (regenerants 140-291), with the exception of regenerants 225 and 245, in which only a fragment length of 650 bp (*nptII* selection gene) was multiplied, the presence of both transgenes (*gfp* and *nptII*) was confirmed. With regenerant 245, a slightly shorter replicate fragment of 650 bp specific to the *nptII* gene was multiplied. This was probably the result of mutation, the deletion of an individual nucleotide or nucleotides. The deletion of embedded transgenes was reported in a small number transformed plants by Hiei *et al*. (1994) in rice, Yao *et al*. (1995) in apple, Mercuri *et al*. (2000) in African violets, Atkinson and Gardner (1991) in *Solanum muricatum* and Atkinson and Gardner (1993) in tamarillo. On the selective medium, 98.7 % of regenerants included both transgenes from the genetic construct, with *gfp* and *nptII* genes, only 1.3 % of the genetic construct with *nptII* gene, (Figure 2 - shows only 20 of 161 regenerants and Table 5). No data were found in the literature on only part-installation of the genetic construct.

In all 9 or 5.4% regenerants of tobacco grown on non-selective medium (regenerants 292-300), the presence of fragment length 422 bp (marker *gfp* gene) and 650 bp (*nptII* selection gene) was found. On the non-selective medium, 3.0% of regenerants failed (Table 3).

Non-expression of the selection transgene, which was observed in regenerants that, due to degradation were transferred from the selective to non-selective medium but the presence was confirmed by molecular analysis, may be the result of installation of the transgene on the range of the plant chromosome that is transcriptionally inactive, mutations or gene silencing. Other authors have also reported that some regenerants that were negative a marker enzyme of β-glucuronidase (GUS), had the presence of the *gus* gene confirmed by hybridization (Hiei *et al*., 1997) or by PCR analysis (Yao *et al*., 1995, Mercuri *et al*., 2000).

4 CONCLUSION

Timentin at a concentration of 150 mg/l completely prevented the growth of *A. t*. LBA4404 bacteria with included plasmid pCAMBIA1390-DsRed or pART27 2mgfp5-ER and had no negative impact on regeneration or the growth and development of regenerants. The success of transformation, with the confirmed presence of both transgenes from the gene construct, both marker *DsRed* gene and selection *hptII* gene, was 93.1 %, while the genetic construct with marker *gfp* gene and selection *nptII* gene was 98.7 %. In regenerant designated 245, the fragment multiplied slightly less than the expected 650 bp specific to the *nptII* gene. This was probably the result of mutation, the deletion of an individual nucleotide or nucleotides. With the transfer of regenerants that had grown poorly on the selective medium to the non-selective medium, we confirmed the non-expression or silencing of selection transgenes, the presence of which was confirmed on the DNA level.

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