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The impact of plasmid on regeneration and expression efficiencies of *gfp* gene in tobacco (*Nicotiana tabacum* L.)

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ABSTRACT

Tobacco leaf explants were transformed by bacteria *Agrobacterium tumefaciens* (*A. t.*) and plasmid pBIN mgfp5-ER, which has a single copy of the green fluorescent *gfp* gene and *A. t.*-pART27 2mgfp5-ER, which has two copies of the *gfp* gene. Both plasmids have a built-in selection *nptII* gene for resistance to the antibiotic kanamycin. The presence of the green fluorescent mGFP-ER protein was detected with the epifluorescent microscope in the individual cells 3 days after transformation with *A. t.*-pART27 2mgfp5-ER and after 6 days in cells transformed with *A.t.*-pBIN mgfp5-ER. After infection by *A. t.*-pART27 2mgfp5-ER, in most cases the regeneration was direct, without intermediate stages of callus and faster, as the first globular structures were formed 10–12 days after transformation and a 204 % regeneration was achieved, while the first globular structure, after infection with *A. t.*-pBIN mgfp5-ER, occurred after 18 days and formed more callus and the regeneration was only 78.4 %. The duplex PCR analysis, performed on all 149 resulting regenerants, confirmed the presence of fragments of length 650 bp specific to the selection *nptII* gene and length of 422 bp specific for *gfp* marker gene.

Key words: *Nicotiana tabacum,* marker gfp gene, selection nptII gene, transformation efficiencies, transgene exspression, DNA analysis

IZVLEČEK

VPLIV PLAZMIDA NA USPEŠNOST REGENERACIJE IN IZRAŽANJA *gfp* GENA V TOBAKU (*Nicotiana tabacum* L.)

Listne izsečke tobaka smo transformirali z bakterijo *Agrobacterium tumefaciens* (*A. t.*) in plazmidom pBIN mgfp5-ER, ki ima eno kopijo zeleno fluorescentnega *gfp* gena in *A. t.*-pART27 2mgfp5-ER, ki ima dve kopiji *gfp* gena. Oba plazmida imata vgrajen še selekcijski *nptII* gen za odpornost na antibiotik kanamicin. Prisotnost zeleno fluorescentnega mGFP-ER proteina smo z epifluorescentnim mikroskopom zasledili v posameznih celicah 3 dni po transformaciji z *A. t.*-pART27 2mgfp5-ER in po 6 dneh tudi v celicah transformiranih z *A. t.*-pBIN mgfp5-ER. Regeneracija je bila po okužbi *A. t.*-pART27 2mgfp5-ER v večini primerov direktna, brez vmesne faze kalusa in hitrejša, saj so prve globularne strukture nastale že 10–12 dni po transformaciji ter dosežena je bila 204 % regeneracija. Prve globularne strukture po okužbi z *A. t.*-pBIN mgfp5-ER so se pojavljale šele po 18 dneh, nastalo je več kalusa in regeneracija je bila nižja, samo78,4 %. Pri vseh 149 nastalih regenerantih smo z dupleks PCR analizo potrdili prisotnost fragmentov dolžine 650 bp, značilnih za selekcijski *nptII* gen in fragmentov dolžine 422 bp, značilnih za markerski *gfp* gen.

Ključne besede: *Nicotiana tabacum*, markerski *gfp* gen, selekcijski *nptII* gen, uspešnost transformacije, izražanje transgenov, DNA analiza

1 INTRODUCTION

Biotechnological techniques of genetic transformation represent an integral complement and an appealing alternative to conventional plant breeding methods, since they enable a relatively rapid introduction of desirable traits into selected cultivars. With the possibility to introduce foreign

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DNA into plant cells, it has become possible to modify the expression of plant endogenous genes or to introduce novel genes of agronomical importance. Genetic transformation has become useful in improving plant properties and for the detection of gene functions in plants (Rao *et al*., 2009).

An efficient plant regeneration system is an important prerequisite for a successful transformation procedure. Test or marker genes are genes whose gene product can be visually identified and is location determined. They enable quick identification of transformed tissues. Marker genes that can be detected by other means, such as taste or smell, can also be useful (Witty, 1989).

In most cases, only a small proportion of plant cells transform, so it is necessary to include a selection gene together with the desired gene, by which transformed cells can be distinguished from non-transformed ones. 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 change and improve (Reichel *et al*., 1996; Haseloff *et al*., 1997).

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 can also be used to monitor the destiny of transgenes introduced into cultivated plants and their impact on the environment (Stewart, 2005).

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).

In this study, we monitored the influence of plasmid on regeneration and phenotypic expression of *gfp* fluorescent genes and selection *nptII* gene in tobacco.

2 MATERIAL AND METHODS

2.1 Plant material, plasmids and agrobacterium-mediated transformation

The leave explants of micropropagated tobacco variety Havana 38 were used for transformation with two plasmids. The commercial bacterium *A. t*. strain LBA4404 contains plasmid pBIN mgfp5-ER or plasmid pART27 2mgfp5-ER. Plasmid pBIN mgfp5-ER is a binary vector, it contains the marker green fluorescent *gfp* gene and the plant selection *nptII* gene for resistance to the amino glycoside antibiotic kanamycin for selection of transformed plant tissues. Plasmid pART27 2mgfp5-ER is a binary vector, which contains two repetitions of mgfp5-ER gene from the vector pBIN mgfp5-ER and the same selection *nptII* gene.

Transformation of tobacco leaves with *A. t*. was performed using a slightly modified method for transformation of leaves as suggested 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 pBIN mgfp5-ER 60 leaf explants were prepared and for plasmid pART27 2mgfp5-ER 50 explants.

Bacterial suspensions of *A. t*., with the appropriate plasmid included, were incubated and prepared for transformation and co-cultivated according to Oven and Luthar (2013). Then, the leaf explants were transferred onto selective *MSr* medium with the addition of [Fe-Na_2-EDTA 0.1 mg/l, thiamine 0.1 mg/l, 6-benzylaminopurine (BAP) 1.0 mg/l, 1-naphthaleneacetic acid (NAA) 0.1 mg/l, agar 8 g/l; pH 5.8] (Stolarz et al., 1991) without acetosyringone and with the addition of timentin 150 mg/l to prevent the growth of *A t*. bacteria and an appropriate selection antibiotic 300 mg/l of kanamycin for the selection of tobacco transformants after infection both with *A. t*.-pBIN

mgfp5-ER or *A. t.*-pART27 2mgfp5-ER. Explants were cultured in a growth chamber at a 16/8 hour photoperiod and at 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 *MS* medium with the addition of the selection antibiotic kanamycin, without timentin. After five weeks, the regenerants that had successfully grown were transferred to the appropriate *MS* selective medium.

2.2 Expression of *gfp* gene

Expression of fluorescent marker genes in the explants was observed 3 and 6 days after infection and 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 green fluorescence *gfp* gene. For the detection of green fluorescent protein mGFP5-ER (both of plasmids pBIN mgfp5-ER or 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.

2.3 Molecular analysis transgenes by PCR method and agarose gel electrophoresis

For determination of the presence of transgenes in 149 tobacco transformed regenerants and non-transformed – negative control, the complete DNA was isolated, according to the method of Kump *et al*. (1992). The concentration of isolated DNA in solution was measured using a DNA fluorimeter DyNA Quant™ 200 (GE Healthcare), according to the standard method of producer. DNA samples were diluted to 20 ng/µl.

Specific multiplication of *gfp* and *nptII* genes was carried out in duplex PCR reactions using two pairs of primers: GFP1a (forward: 5'-AGT GGA GAG GGT GAA GGT GAT G-3') / GFP1b (reverse: 5'-TTG TGG CGG GTC TTG AAG TTG G-3') and NPTII1a (forward: 5'-GAG GCT ATT CGG CTA TGA CTG-3') / NPTII1b (reverse: 5'-ATG GGG AGC GGC GAT ACC GTA-3'). In a total volume of 25 µl the PCR reaction mixture contained 5 µl of DNA and 20 µl of PCR mixture: 1×PCR buffer [10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40] (Fermentas), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 4×0.5 µM suitable primer and 0.5 units of enzyme Taq DNA polymerase (Fermentas) were added. 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 analysis amplified fragments by agarose gel electrophoresis.

For the separation of DNA fragments, horizontal electrophoresis was used on a 1.4 % gel according Oven and Luthar (2013).

3 RESULTS AND DISCUSSION

3.1 Regeneration of tobacco leaf explants and transgene expression

After three days of *A. t.*-pART27 2mgfp5-ER transformation, some cells expressed the mGFP5-ER protein at the leaf explants and after 6 days, the mGFP5-ER protein expression in the cells transformed with *A. t.*-pBIN mgfp5-ER (Figure 1).

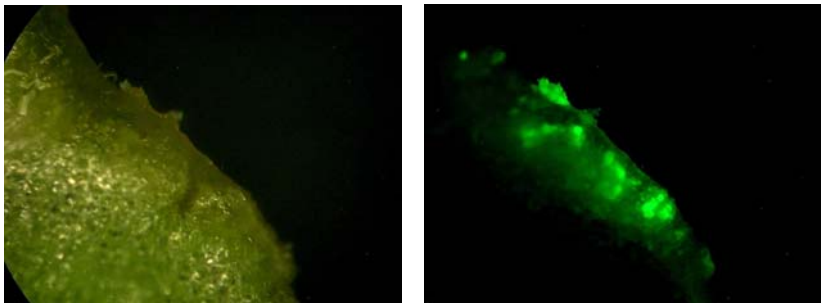

Figure 1: Observation of the mGFP5-ER protein expression after 6 days *A. t.* mediated transformation of tobacco examined under an epifluorescence microscope with white light (left) and with the special filter set for detection of green fluorescence (right)

Germs of the first regenerants occurred after 10-12 days after transformation with *A. t.*-pART27 2mgfp5-ER and after 18 days after transformation with *A. t.*-pBIN mgfp5-ER. After transformation *A. t.*-pART27 2mgfp5-ER the regeneration was mostly direct, without an intermediate callus (Figure 2), as noted by Stolarz *et al*. (1991). After transformation with *A. t.*-pBIN mgfp5-ER we obtained more callus and less regenerants.

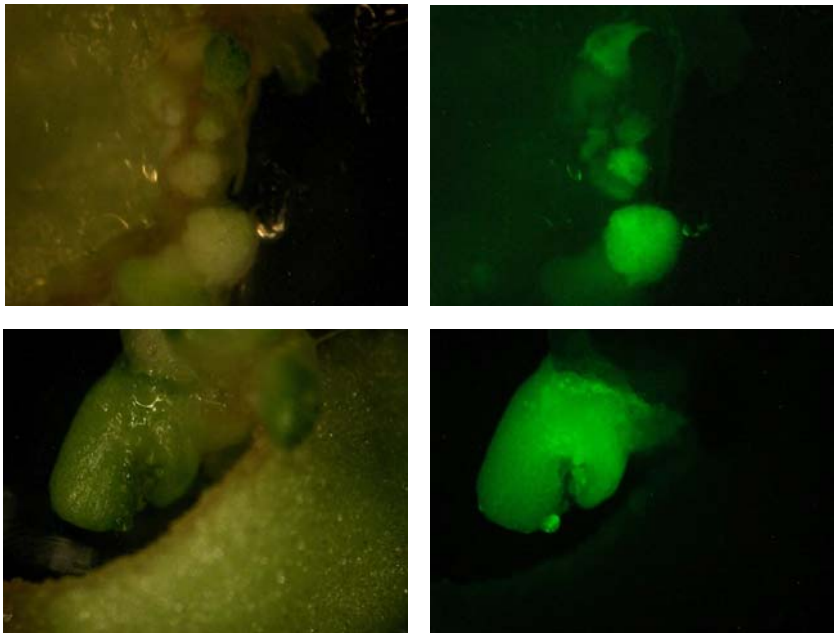

Figure 2: Observation of the mGFP5-ER protein expression in globules and regenerant after *A. t.* mediated transformation of tobacco examined under an epifluorescence microscope with white light (left) and with the special filter set for detection of green fluorescence (right)

After five weeks, a large number of regenerants was observed after transformation with *A. t.*-pART27 2mgfp5-ER and less regenerants after transformation with *A. t.*-pBIN mgfp5-ER. Regenerants from leaf explants, in which phenotypic expression of the inserted fluorescent genes was observed, were transferred onto *MS* medium with the addition of an appropriate selection antibiotic. After next five weeks we obtained new regenerants. In total, 102 regenerants were obtained from 50 explants after transformation with *A. t.*-pART27 2mgfp5-ER and less, only 47 regenerants from 60 explants after transformation with *A. t.*-pBIN mgfp5-ER.

The 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 in the combination with plasmid pART27 2mgfp5-ER.

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.

3.2 Molecular analysis of transgenes integration

DNA analysis was performed on all 149 surviving regenerants.

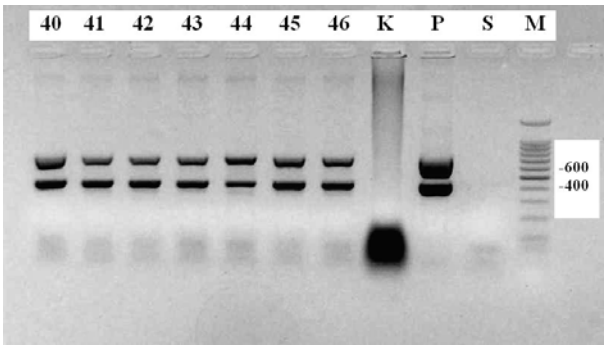

Figure 3: Amplified DNA fragments by duplex PCR with the specific set of primers for the *mgfp-ER* gene (422 bp) and the specific set of primers for the *nptII* gene (650 bp). The figure shows only the 7 regenerants of 149. 40 – 46: transformed tobacco regenerants; K: control, non–transformed tobacco; P: plasmid; S: blind samples; M: size standard.

In all 149 regenerants of tobacco transformed with *A. t*.-pBIN mgfp5-ER (47) and with *A. t*.-pART27 2mgfp5-ER (102) that were grown on selective medium, the presence of fragment length 650 bp (selection *nptII* gen) and fragment length 422 bp (marker *gfp* gene) was released (Figure 3). The transformation efficiencies achieved 204 % after the *A. t*.-pART27 2mgfp5-ER, and 78.4 % after the *A. t*.-pBIN mgfp5-ER-mediated transformation.

4 CONCLUSION

As a result of transformation with *A. t*.-pART27 2mgfp5-ER the regeneration capacity was faster and efficient, mostly direct, without an intermediate callus, while after transformation with *A. t*.-pBIN mgfp5-ER more callus and 2.6 times less regenerants were obtained.

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