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FEASIBILITY OF HYGROMYCIN AS A SELECTION AGENT IN *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF OILSEED RAPE (*BRASSICA NAPUS* L.)

*Tímea Kuťka Hlozáková[1], Zuzana Polóniová[2], Jana Moravčíková[2]**

Address(es): Ing. Jana Moravčíková, PhD.,
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Science, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, phone number: 037/ 641 4697.
[2]Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, P.O.Box 39A, 95007 Nitra, Slovakia.

*Corresponding author: jana.moravcikova@savba.sk

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ABSTRACT

In this work the feasibility of the antibiotic hygromycin as a selection agent in *Agrobacterium*-mediated transformation of oilseed rape (*Brassica napus* L.) was evaluated. For this, two economically important commercial varieties Haydn and Hunter and tobacco as a model plant were subjected to *Agrobacterium*-mediated transformation. The 5-6 days-old oilseed rape hypocotyls and 4-6 weeks-old tobacco leaf segments were transformed with the binary vector pCambia1304. The T-DNA contained the reporter *gfp:gus* and the selectable marker *htp* genes. Regeneration of transformed cells was conducted under selection of 10 mg.l^{-1} (oilseed rape) and 30 mg.l^{-1} (tobacco) hygromycin. Putative transgenic plantlets were analysed by the mean of the histochemical GUS and PCR analyses. Transformation efficiency ranged from 1.0% (cv. Haydn) to 40.4% (tobacco). No transgenic shoots were detected for the cv. Hunter. It points out the oilseed rape cultivar specificity plays significant role in choice of suitable selection agent.

Keywords: *Agrobacterium tumefaciens*, *Brassica napus* L., β-glucuronidase gene, hygromycin, oilseed rape, tobacco

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is the second most commonly grown oilseed crop in the world production after soybean. Most of global production is represented by the cultivars of so-called "Canola" with reduced content of erucic fatty acid and the glucosinolates. The first transgenic oilseed rape plants were prepared more than 25 years ago **(Fry *et al*., 1987; Pua *et al.*, 1987)**. However, commercialisation of transgenic oilseed rape plants is slow. Until now, only Argentine Canola genetically modified for herbicide tolerance in combination with male sterility has been released into the environment and authorised as a food and feed (GM Crop Database, http://cera-gmc.org). In laboratory, transgenic shoots were obtained from different types of explants such as cotyledons **(Moloney *et al*., 1989)**, hypocotyls **(Radke *et al*. 1988; Wang *et al*., 2010)**, the intermodal segments **(Fry et al., 1987)**, microspores **(Abdollah *et al*., 2009)** or the flower stems **(Boulter *et al*., 1990)**. Several delivery methods including *Agrobacterium*-mediated transformation **(Moloney *et al*., 1989),** microinjection **(Spangenberg *et al*., 1986)**, electroporation **(Guerche *et al*., 1987)** or particle bombardment **(Abdollahi *et al.*, 2011)** have been applied with variable success. However, it has been found the regeneration of oilseed rape is variable and genotype specific **(Tang *et al*., 2003; Kamal *et al*., 2007; Khan *et al*., 2010; Bhowmik *et al*., 2011; Boszoradova *et al*., 2011)**. For this reason, the majority of transformation studies have been performed with a limited number of cultivars, mainly with the cv. Westar.

A low efficiency of plant transformation necessitates the use of an appropriate selection system. Plant susceptibility to antibiotics change broadly among species, genotypes and plant tissues **(Padilla and Burgos, 2010)**. Excessively high antibiotic concentration may kill non-transformed cells; thereby inhibiting regeneration of transformed ones **(Escandon and Hahne, 1991)**. In contrast, insufficient level of antibiotics may results in occurrence of many escapes and chimeras, thus inhibiting the regeneration and effective selection of transformed cells. In oilseed rape transformation, the most common regeneration was performed under selection of antibiotic kanamycin **(Moloney *et al*., 1989; Wallbraun *et al*., 2009; Boszoradova *et al*., 2011)**. The concentration varied in dependence on the explant type and variety used. For example, **Wallbraun *et al.* (2009)** used for regeneration of transformed hypocotyls (cv. Dakkar) kanamycin at concentration of 50 mg.l^{-1}, while **Boszoradova *et al.* (2001)** regenerated transformed cotyledonary petioles (cv. Topas) at 10 mg.l^{-1} kanamycin.

In this work we tested the feasibility of the antibiotic hygromycin as a selection agent in oilseed rape transformation. For this, two economically important commercial varieties Haydn and Hunter and tobacco as a model plant were subjected to *Agrobacterium*-mediated transformation. The regeneration potential of transformed cells under selection pressure of hygromycin was evaluated.

MATERIAL AND METHODS

Bacteria and vector construct

In plant transformation experiments, the binary vector pCambia 1304 (http://www.cambia.org/daisy/cambia/585) was used. The T-DNA of the plasmid pCambia 1304 contained the *mgfp*5 version of the *Aequoria victoria* green fluorescent protein in translational fusion with β-glucuronidase (*gus); and* the selectable marker hygromycin phosphotransferase (*hpt*) genes (Figure 1). The plasmid pCambia 1304 was introduced into *Agrobacterium tumefaciens* strain LBA 4404 and its stability was verified by restriction analyses after retransformation into *E. coli*.

Bacterial cells were grown in Luria and Bertani (LB) medium **(Sambrook *et al*., 1989)** containing 25 mg.l^{-1} rifampicin and 50 mg l^{-1} kanamycin. To prepare *Agrobacterium* inoculum, an overnight bacterial culture was centrifuged at 4000 rpm for 10 min and the cells were resuspended in 20 ml of the liquid MS medium **(Murashige and Skoog, 1962)** medium to the optical density OD_{600} of 0.6.

Plant material and transformation

Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) and the two spring oilseed rape (*Brassica napus* L.) cultivars Hunter and Haydn have been used. The cultivar Hunter was obtained from Raps GbRSaatzuchtLundsgaard, Germany and the cv. Haydn from NorddeutschePflanzenzucht, Hans-Georg Lembke KG, Holtsee, Germany.

Seeds were surface-sterilized with 96% (v/v) ethanol, washed in 10% (v/v) sodium hypochloride and 0.1% (v/v) Tween 20, and rinsed five times in the sterile distilled water. The seeds were germinated on the MS medium with 2% (w/v) sucrose, solidified with 0.7% (w/v) plant agar pH 5.8, at 25°C and 16 h/ 8 h light/dark photoperiod under 50 μE.m^{-}2.s^{-1} light intensity.

The 4-6 weeks old tobacco plants were transformed using the leaf disc transformation protocol described by **Horsch *et al.* (1985)**. The transformed tobacco tissue was selected on medium with 30 mg.l^{-1} hygromycin.

The 5-6 days old hypocotyls of the oilseed rape cultivars were transformed according to the protocol by **Boszoradova *et al.* (2011)**. During the first two

weeks the transformed oilseed rape tissues were regenerated without selection pressure and afterwards at a concentration of 5 mg.l^{-1} hygromycin. Two weeks later, the tissues were regenerated in the presence of 10 mg.l^{-1} hygromycin.

β-glucuronidase assays

Histochemical GUS assay was conducted as described by **Jefferson *et al.* (1987)**. Leaf explants were incubated in 1 mmol.l^{-1} 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Duchefa, Netherlands), 50 mmol.l^{-1} phosphate buffer (pH 7) at 37°C in the dark overnight. To improve colour contrast, the tissues were washed in 70% (v/v) ethanol.

PCR analyses

Genomic DNA was isolated from the tobacco and oilseed rape leaf tissues using DNeasyPlant Mini kit (Qiagen). PCR primers for detection of the *gfp:gus* genes were P1 (5'-TTCAAGACCCGCCACAACATCGAA-3') and P2 (5'-ATTCACCACTTGCAAAGTCCCGCT -3') for detection of the *htp* gene were P3 (5'- AGCTGCATCATCGAAATTGCCGTC-3') and P4 (5'-ATAGCTGCGCCGATGGTTTCTACA -3'). The PCR reaction was performed in 50 µl mixture containing 100-200 ng of DNA template, 15 pmol of each primer, 200 µM dNTPs, 1 × PCR buffer, 2.5 mmol l^{-1} $MgCl_2$ and 1 unit of FIREPol DNA polymerase (Solid Biodyne, Estonia). The first PCR step of 95°C for 3 min was followed by 35 cycles of 95°C for 30s, 60°C for 45 s and 72°C for 1 min. The last step was performed at 72°C for 10 min.

RESULTS AND DISCUSSION

In this work we evaluated the potential of antibiotic hygromycin as a selection agent in *Agrobacterium*-mediated transformation of the two oilseed rape cultivars Haydn and Hunter. As a control, tobacco was used. Tobacco is considered a model plant for *Agrobacterium*-mediated transformation **(Ganapathi *et al.*, 2004).** The oilseed rape seeds were germinated under dark conditions. Before transformation experimnets, hypocotyl segments were pre-conditioned in liquid callus-inducing medium. Such treatment could help to overcome necrosis after transformation **(Cardoza and Stewart, 2003)**. Tobacco seeds were germinated under standard *in vitro* conditions without any pre-conditioning.

To find out selection pressure, non-transformed 5-6 days-old oilseed rape hypocotyl and 4-6 weeks old leaf segments were allowed to regenerate in the presence of different concentration of hygromycin. Based on these results (data not shown), the concentrations of hygromycin10 mg.l^{-1} and 30 mg.l^{-1} were chosen as the selective agent for oilseed rape and tobacco explants (respectively). Subsequently, the hypocotyl (oilseed rape) and leaf (tobacco) segments were transformed with *A. tumefaciens* LBA 4404 carrying the binary vector pCambia 1304. The T-DNA contained selectable marker *htp* gene conferring resistance to the antibiotic hygromycin B and the reporter *gfp:gus* genes (Figure 1). To increase the recovery of transgenic shoots, transformed oilseed rape hypocotyls were cultured for 14 days on the media without antibiotic hygromycin and then in the presence of hygromycin at concentration of 5 mg.l^{-1}. Two weeks later, the concentration was increased to 10 mg.l^{-1}. The application of antibiotics at lower concentrations and/or postponing of the selection pressure several days after *Agrobacterium* infection could support transformed cells to grow and develop (**Pandian *et al.*, 2006; Boszoradova *et al.*, 2011)**. Under given conditions, the first shoots appeared after 4-6 weeks. The efficiency of shoot formation varied. The lowest (12.3%) was observed in the cv. Hunter, while cv. Haydn produced shoots with 24.3% and tobacco with 63.6% efficiencies. Data are summarised in Table 1. To evaluate the presence and activity of the *gus* reporter gene, all regenerated shoots were analysed histochemically with X-gluc as a substrate for the activity of the enzyme β-glucuronidase (**Jefferson *et al.*, 1987)**. An example of the histochemical GUS activity assay is given in Figure 2. Our results showed that no all shoots were histohemically GUS-positive. Under given selection pressure only 4.3% (cv. Haydn) and 63.5% (tobacco) analysed shoots were GUS-positive. Contrary, no GUS-positive shoots were detected in cv. Hunter. Such escapes could coincide with weaker selection pressure **(Padilla and Burgos, 2010)**. The transformation efficiency was expressed as the number of GUS-positive shoots obtained as percentage of the number of explants used. It varied from 0% (cv. Hunter), 1.0% (cv. Haydn) to 40.4% (tobacco). Several factors, such as plant species, genotypes, explant types, medium composition, bacterial strains or selection conditions could influence transformation efficiencies. For example, **Boszoradova *et al.* (2011)** used the same varieties (Hunter and Haydn) but the regeneration of transformed cells was performed under selection pressure of kanamycin. They reported the transformation efficiencies of 2.9% and 2.0% for the cultivars Hunter and Haydn (respectively).

The transgenic nature of the GUS-positive plants was also confirmed by PCR analyses. The primer sets P1/P2 and P3/P4 were designed to amplify 939 bp and 710 fragments corresponding to the chimeric *gus:gfp* and *htp* genes (respectively). The PCR product was detected in all analysed GUS-positive oilseed rape and tobacco plants. No amplicon was detected in non-transformed control as well as in histochemically GUS-negative plants (Figure 3).

Table 1 Summary of plant transformation experiments

	cultivar	Explant type	No. explants [a]	No GUS (+) shoots [b]	Transformation efficiency (%)[c]
oilseed rape	Hunter	hypocotyls	244	0 (30)	-
	Haydn	hypocotyls	288	3 (70)	1.0
tobacco	Petit Havana	leaf discs	99	40 (63)	40.4

[a] Number of explants used in transformation experiments

[b] Number of shoots GUS positive in the histochemical assay. In brackets, total number of shoots regenerated under selection pressure of 30 mg.l^{-1} (tobacco) and 10 mg.l^{-1} (oilseed rape) hygromycin is given.

[c] Transformation efficiency expressed as the number of GUS(+) shoots obtained as percentage of the number of explants used.

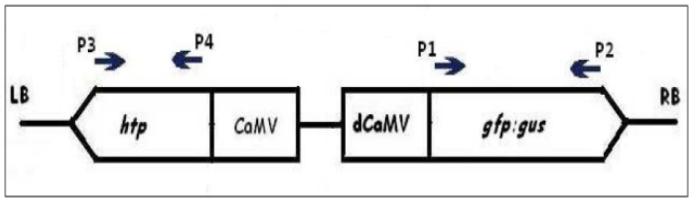

Figure 1 The T-DNA structure of the plant binary vector pCambia 1304.
The T-DNA consists of the reporter (*gus:gfp*) genes under control of the double CaMV 35S promoter (*dCaMV35S*), and the hygromycin phosphatase gene (*htp*) regulated by the CaMV 35S promoter. All genes are terminated by the *nos* terminator. The sets of primers used for PCR analyses are indicated as P1/P2 and P3/P4. Other abbreviations used: RB, LB – right and left borders of T-DNA

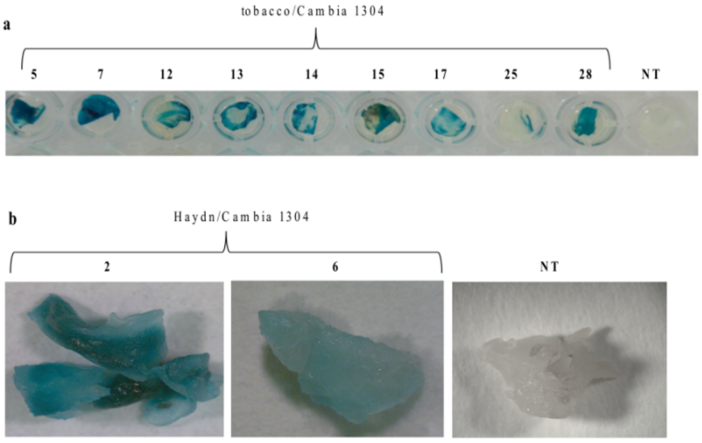

Figure 2 Histochemical detection of the GUS activity on tobacco **(a)** and oilseed rape **(b)** tissues/pCambia 1304.
GUS activity was detected as a blue color at the place of the enzymatic reaction. NT – non transformed tissues.

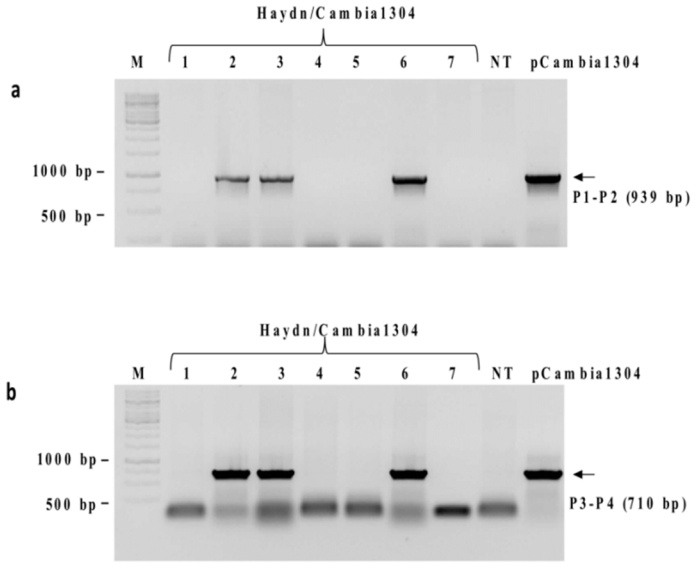

Figure 3 Photographs of ethidium bromide-stained 1% agarose gels with PCR products obtained on transgenic T_0 oilseed rape.
(a) PCR results with the primers P1/P2 that amplified an internal 939 bp fragment of the *gus:gfp* genes. **(b)** PCR results with the primers P3/P4 that amplified an internal 710 bp fragment corresponding to the *htp* gene. The lane M contains 1 kb DNA ladder (Fermentas) as a size marker, lanes 1-7 putative transgenic oilseed rape plantlets, NT – non-transformed oilseed rape plant, pCambia 1304 – plasmid pCambia 1304 used for plant transformation.

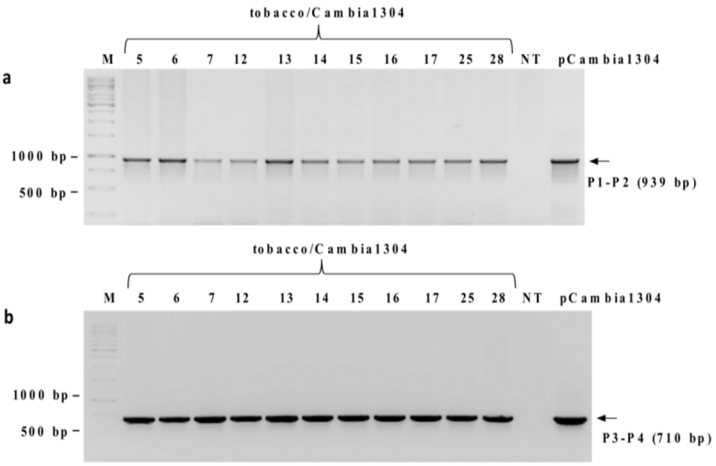

Figure 4 Photographs of ethidium bromide-stained 1% agarose gels with PCR products obtained on transgenic T_0 tobacco. (**a)** PCR results with the primers P1/P2 that amplified an internal 939 bp fragment of the *gus:gfp* genes. (**b)** PCR results with the primers P3/P4 that amplified an internal 710 bp fragment corresponding to the *htp* gene. The lane M contains 1 kb DNA ladder (Fermentas) as a size marker, lanes 5-28 putative transgenic tobacco plantlets, NT – non-transformed tobacco plant, pCambia 1304 – plasmid pCambia 1304 used for plant transformation.

CONCLUSION

With the aim to test the feasibility of the antibiotic hygromycin as a selection agent in oilseed rape transformation, two oilseed rape cultivars Hunter and Haydn as well as tobacco as a model plant were subjected to A*grobacterium* infection. Transformed cells were regenerated under selection pressure of hygromycin at concentrations of 10 $mg.l^{-1}$ (oilseed rape) and 30 $mg.l^{-1}$ (tobacco). Under given conditions, transgenic shoots were generated from the oilseed rape cv. Haydn with 1.0% and from the tobacco plants with 40.4% efficiencies. However, no transgenic shoots were generated from the cv. Hunter. It points out the oilseed rape cultivar specificity play significant role in choice of suitable selection agent.

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