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

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ABSTRACT

In this work we tested 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 hph 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://gm-crop.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 (Boultet et al., 1990). Several delivery methods including Agrobacterium-mediated transformation (Moloney et al., 1989), microinjection (Spangenberg et al., 1986), electroporation (Guérche 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; Bhownik 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 result 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

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 mpg5 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 OD₅₆₅ of 0.6.

Plant material and transformation

Tobacco (Nicotiana tabacum cv. Petiti 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 GbR Saatzuchten, Undsgaard, Germany and the cv. Haydn from NorddeutschePflanzenzucht, Hans-Georg Lembcke 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.

\section*{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.

\section*{Genomic DNA isolation}

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 P\(_1\) (5'-TTCAAGACCCCGCCACAAATCGAA-3') and P\(_2\) (5'-ATTCCACATTGCAAAATCCGGCT-3') for detection of the hpt gene were P\(_3\) (5'-AGTGCACTATCGAAATTCCGTC-3') and P\(_4\) (5'-ATAGGTGCGCCGATGGTTCTACA-3'). The PCR reaction was performed in 50 µl mixture containing 100-200 ng of DNA template, 15 pmol of each primer, 200 µg dNTPs, 1 × PCR buffer, 2.5 mmol L\(^{-1}\) MgCl\(_2\) and 1 unit of FIREPol DNA polymerase (Solida 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 1 min and 72°C for 1 min. The last step was performed at 72°C for 10 min.

\section*{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 organism for Agrobacterium-mediated transformation (Gosnati et al., 2004). The oilseed rape seeds were germinated under dark conditions. Before transformation experiments, 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 hygromycin 10 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 hpt gene conferring resistance to the antibiotic hygromycin B and the reporter gfp::gus genes (Figure 1). To decrease 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 postponing of the selection pressure several days after Agrobacterium infection could support transformed cells to grow and develop (Padilam 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 histochemically GUS-positive. Under given selection pressure only 4.3% (cv. Haydn) and 63.5% (tobacco) analysed shoots were GUS-positive. In contrary, no GUS-positive shoots were detected in cv. Hunter. Such escapes could coincide with weak 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 hpt 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).

\begin{table}
\begin{center}
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{culti\textbar{}var} & \textbf{Explant type} & \textbf{No. explants} & \textbf{No. GUS(+) shoots} & \textbf{Transformation efficiency (%)} \\
\hline
oilseed rape & Hunter & hypocotyls & 244 & 0 (30) & - \\
Haydn & hypocotyls & 288 & 3 (70) & 1.0 \\
Petit Havana & leaf discs & 99 & 40 (63) & 40.4 \\
\hline
\end{tabular}
\end{center}
\caption{Summary of plant transformation experiments}
\end{table}

\begin{itemize}
\item Number of explants used in transformation experiments
\item 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.
\item Transformation efficiency expressed as the number of GUS(+) shoots obtained as percentage of the number of explants used.
\end{itemize}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{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 (dCaMV/35S), and the hygromycin phosphatase gene (hpt) 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 – right and left borders of T-DNA}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{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.}
\end{figure}
Figure 3 Photographs of ethidium bromide-stained 1% agarose gels with PCR products obtained on transgenic T1 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 hpt 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.

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 Agrobacterium 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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