

## A transformation method for obtaining marker-free plants based on phosphomannose isomerase

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### Abstract

A selectable marker system for plant transformation of rape plants that does not require the use of antibiotics or herbicides was developed. The selectable marker consists of the phosphomannose isomerase (PMI) gene from *E. coli* under the control of a plant promoter. The PMI gene was transferred to *Brassica napus* by *Agrobacterium*-mediated transformation, which allows the selection of transgenic plants with mannose and sucrose as selective agents. The highest transformation frequency of 7.9 % was obtained when a combination of 4.5 g l<sup>-1</sup> mannose and 10 g l<sup>-1</sup> sucrose was used. For early identification of transgenic events, histochemical staining with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-Gluc) was used. Stable integration of the transgene was confirmed by PCR and Southern blot analysis. These preliminary results indicate that the mannose selection system can be used for *Agrobacterium*-mediated transformation of rape plants.

**Key words:** *Agrobacterium tumefaciens*, mannose selection marker, rape, transformation.

### Introduction

The use of selection-marker genes in genetic transformation protocols has been developed to limit the regeneration of non-transgenic shoots after transformation. The majority of existing selection systems are based on antibiotic or herbicide resistance.

Regulatory committees and the public worldwide debate the safety of these markers in genetically modified food organisms. In order to turn the negative debate against Genetic Modified Organisms it is important to find alternatives to the use of antibiotics as selection agents.

One potential alternative is provided by the so-called positive selection systems, where the selective agent is converted into fully metabolised compounds (Joersbo 2001). One of these systems uses the phosphomannose isomerase (PMI), derived from *Escherichia coli* (Miles, Guest 1984), to convert mannose-6-phosphate to fructose-6-phosphate. Only transformed cells are capable of utilising mannose as a carbon source.

PMI has been used as a selectable marker for transformation of many plant species, such as sugar beet (Joersbo et al. 1998), maize (Negrotto et al. 2000; Wright et al. 2001), wheat (Reed et al. 2001), rice (Lucca et al. 2001) and pearl millet (O'Kennedy et al. 2004).

We report here on the use of mannose as a selectable agent for the recovery of transgenic rapeseed via *Agrobacterium tumefaciens*-mediated transformation. This is the first report of this system used in *B. napus* plants.

## Materials and methods

Hypocotyl segments of *in vitro*-germinated plants of rape (*Brassica napus* Mill.) cv. 'Drakkar' were inoculated with the construct pNOV-GUS provided by Syngenta. The GUS intron gene (Vancanneyt et al. 1990) was cloned into the *Hind*III site of pNOV2819. The experimental design was completely randomized with two replications, each consisting of a minimum of 15 petri dishes (25 explants per dish). After three days of co-cultivation, the hypocotyl explants were subsequently selected on Murashige and Skoog (1962) medium supplemented with five combinations with different concentrations of mannose and sucrose (Table 1).

All selection media contained 500 mg l<sup>-1</sup> carbenicillin in order to eliminate *Agrobacterium* contamination. Cultures were maintained under a 16/8-h photoperiod. The frequency of transformation was evaluated using a GUS assay. Leaf segments of regenerated plants were incubated in X-Gluc and GUS-extraction buffer at 36 °C overnight according to Jefferson (1987). The leaves were bleached with several washes of 70 % EtOH. Evaluation was based on the colour reaction. Transgenic plants were confirmed by PCR detection of the PMI gene. PMI-specific primers and the PCR protocol were described by Negrotto et al. (2000). For Southern blotting, about 2.5 µg of DNA were fragmented with *Eco*RV and an electrophoretically chemiluminescent detection system (CDP-Star) according to instruction of the manufacturer (Roche, Mannheim, Germany).

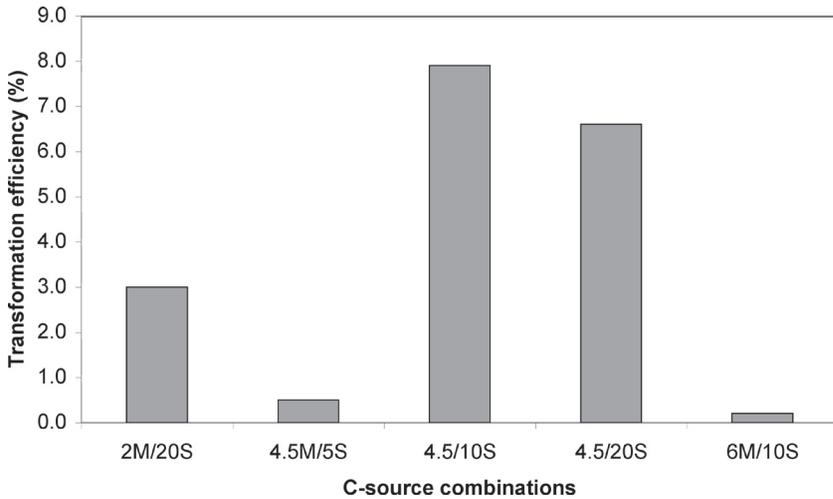
## Results and discussion

Using traditional transformation protocols, plant cells or tissues are placed on culture media containing salts, hormones and carbon source (usually sucrose). For the PMI/mannose selection system plant explants are cultivated on a similar medium supplemented with both sucrose and mannose. This selection system is based on its ability to inhibit *in vitro* organogenesis when non-transformed explants are cultured in medium using mannose as a carbon source.

The transformation frequency that we obtained using the PMI/mannose system in rapeseed transformation is similar to that obtained by traditional selection systems.

**Table 1.** Combinations of mannose and sucrose in the Murashige and Skoog medium used in the experiments

Combinations	Mannose (M)/ Sucrose (S) (g l <sup>-1</sup> ) for the first 6 weeks	Mannose (M)/ Sucrose (S) (g l <sup>-1</sup> ) for the last 2 weeks
1	2 M / 20 S	4.5 M / 5 S
2	4.5 M / 5 S	4.5 M / 10 S
3	4.5 M / 10 S	4.5 M / 10 S
4	4.5 M / 20 S	4.5 M / 10 S
5	6 M / 10 S	6 M / 50 S



**Fig. 1.** Transformation frequencies (calculated as the number of GUS- and PCR-positive shoots relative to the number of inoculated hypocotyl explants) obtained at various combinations of mannose and sucrose in the MS medium. S, sucrose; M, mannose ( $\text{g l}^{-1}$ ).

Screening of various combinations of mannose and sucrose indicated that  $4.5 \text{ g l}^{-1}$  mannose in combination with  $10.0 \text{ g l}^{-1}$  sucrose for the full period in selection medium attained the highest transformation frequency of 7.9 % (Fig. 1). The interaction of sucrose with mannose toxicity was studied at three levels of mannose: 2.0, 4.5, 6.0  $\text{g l}^{-1}$ , respectively. Different levels of sucrose and mannose were used during selection and regeneration, but in the combination where the highest transformation frequency was obtained, the same level of mannose and sucrose was used in all stages. If the mannose level was higher than  $5 \text{ g l}^{-1}$ , the number of regenerated shoots was dramatically decreased. In this study, sucrose was found to strongly influence the transformation frequency, resulting in more than a 10-fold difference if 5 to  $20 \text{ g l}^{-1}$  sucrose was used. Similar observations were made with mannose selection for sugar beet transformation (Joersbo 1999) and with galactose selection for potato transformation (Joersbo et al. 2003).

PCR with *manA*-specific primers detected a fragment with a size of 550bp and Southern blot analysis showed at least one copy of the PMI gene in the transgenic plants.

These results demonstrate that rapeseed explants were able to utilize mannose as a carbon source via the integration of the PMI gene. The development of calli and shoots is comparable to using an antibiotic resistance marker. This first report on the use of the mannose selection system for the production of transgenic rapeseed plants indicates that PMI/mannose is an efficient selection system. Moreover, no potential for risk to animals, humans or environmental safety is known for this method of gene transformation. Further experiments should be carried out in combination with the gene of interest and to further increase the transformation frequency.

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