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Mannose-Based Selection with Phosphomannose-Isomerase (*PMI*) Gene as a Positive Selectable Marker for Rice Genetic Transformation

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Abstract

A positive selectable marker system was adapted for transformation of mature embryo-derived calli of Indica rice (*Oryza sativa* L.) utilizing the *PMI* gene encoding for phosphomannose-isomerase that converts mannose-6-phosphate to fructose-6-phosphate. The transformed cells grew on medium supplemented with 3% mannose as carbon source and calli were selected on media containing various concentrations of mannose. Molecular analyses showed that the transformed plants contained the *PMI* gene. The results indicate that the mannose selection system can be used for *Agrobacterium*-mediated transformation of mature embryo in rice to substitute the use of conventional selectable markers in genetic transformation.

Key words: Genetic transformation, *Oryza sativa* L., Phosphomannose-isomerase, Positive selection, selectable marker gene.

Introduction

Plant genetic transformation technologies rely upon the selection and recovery of transformed cells. Selectable marker genes so far have been either antibiotic resistance genes or herbicide tolerance genes. There is a need to apply alternative principles of selection, as more transgenic traits have to be incorporated into a transgenic crop and because of concern that the use of conventional marker genes may pose a threat to humans and environment. New classes of marker genes are now available conferring metabolic advantage to the transgenic cells over the non-transformed cells (Joersbo 2001; Suprasanna et al. 2002).

Several positive selection systems, based on benzyl adenine N-3-glucuronide, xylose and mannose, have been used to avoid the use of antibiotics or herbicides (Joersbo 2001). Mannose, a hexose sugar, has been known for a number of years to be unable to sustain growth of various plant species (Malca et al. 1967). The *PMI* gene (*PMI*: *manA* from *E. coli*) encoding mannose-6-phophate isomerase has been used as the selectable marker gene since this enzyme converts mannose-6-phosphate to fructose-6-phosphate, which is easily metabolized through gluconeogenesis. Consequently, cells transformed with the *PMI* gene gain a metabolic advantage, compared with the non-transgenic cells that remain unable to metabolize mannose-6-phosphate. The accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, causing a block in glycolysis. In addition, the synthesis of mannose-6-phosphate depletes cells of orthophosphate which is required for ATP production, so the addition of sucrose or glucose could alleviate the effect of mannose on growth and germination (Joersbo et al. 1998).

The structural gene, *manA*, from *Escherichia coli* has been successfully applied to produce transgenic plants of several plant species including sugarbeet (Joersbo et al. 1998, 1999), maize (Negrotto et al. 2000; Wang et al. 2000), wheat (Wright et al. 2000), cassava (Zhang et al. 2000), pearl millet (O'Kennedy et al. 2004), papaya (Zhu et al. 2005), and flax (Lamblin et al. 2007). This selectable marker gene has been reported to be superior to antibiotic or herbicide (pat or bar) selectable marker genes for plant transformation. Herein, we report the use of *PMI* as a selectable marker for *Agrobacterium*-mediated transformation of mature embryo derived calli of *indica* rice.

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Materials and Methods

Rice (*Oryza sativa* L. ssp. *indica*) var. Pusa Basmati 1 was used in this study. Callus cultures were initiated from mature seeds as per the method described earlier (Suprasanna et al. 1995). Mature seeds were disinfected with 0.1% (w/v) $HgCl_2$ for 6-7 min followed by rinsing several times with sterilized water. The embryos were then placed on callus induction (Murashige and Skoog 1962) medium (CIM) supplemented with 2 mg/l 2,4-D, 50 mg/l L-tryptophan and 3% sucrose gelled with 0.2% Gelrite at pH 5.8. Cultures were incubated in the dark at 26±1 °C for 7-14 days for the induction of the embryogenic calli. Embryogenic calli were subcultured on the fresh CIM and kept in the dark for another 4-5 days before being used for transformation. To determine the effects of mannose on germination and callus induction, 24 mature seeds were germinated on MS basal medium supplemented with 3% (W/V) sucrose or mannose, and mature embryo-derived callus (150 mg), was placed on CIM supplemented with either mannose (1-4%) or sucrose (3% W/V). In another experiment, different sugars (3% W/V each of sucrose, glucose, maltose and mannose, and 3% mannose + 0.5% sucrose) were compared for their effects on callus growth by taking 150 mg initial callus and checking the final fresh weights after 2 and 4 weeks.

Bacterial Strain and Plasmid, Transformation

The plasmid pNOV2819 (Fig.1, provided by Syngenta Corporation, Switzerland) was introduced into *A. tumefaciens* EHA105 and EHA105 containing pNOV2819 was inoculated in liquid YENB medium supplemented with 50 mg/l spectinomycin and grown at 28 °C for 24 h. For comparison, transformation was also done with EHA105 containing p1301 containing hygromycin phosphotransferase as a selectable marker gene.

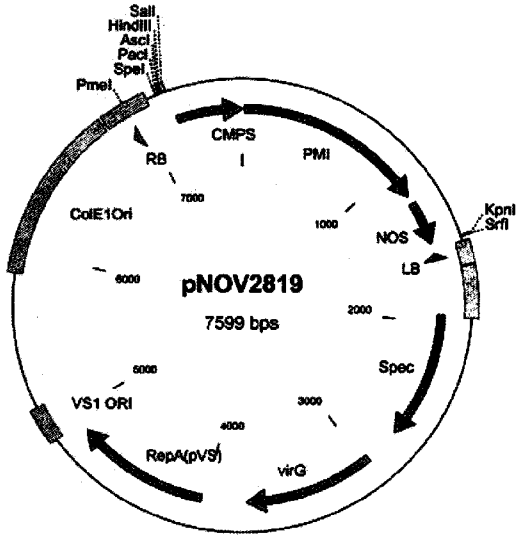

Fig. 1. Plasmid map of pNOV2819 (kindly provided by Syngenta). The *pmi* gene is under the constitutive CMPS promoter (cestrium yellow leaf curling virus promter short version). LB-left border sequence, RB-right border sequence, *pmi-phosphomannose isomerase* gene

The bacteria were suspended in MS basal medium supplemented with 100 mM acetosyringone. The pre-cultured calli were transferred into *Agrobacterium* suspension and soaked for 20 min under continuous shaking (25 rpm) in a sterile flask and then dried on a pad of sterile tissue paper to remove excess surface water. The calli were plated on co-cultivation medium and cultured in the dark for 72 h. The co-cultivated calli were then transferred to the CIM and cultured in the dark at 26±1 °C for 3-4 weeks. For selection, calli were transferred to selection medium (CIM) added with 3% mannose and 0.5% sucrose or 50 mg/l hygromycin. The mannose-resistant calli were transferred to regeneration medium (Suprasanna et al. 1995) and cultured under a 16/8-h (light/dark) photoperiod at 26±1 °C.

Molecular Analysis

For PCR analysis, genomic DNA was extracted from the transformed and non-transformed control lines as per the CTAB method. The PCR amplification was performed in 50 µl reaction volume containing 100 ng of genomic DNA, 1x PCR buffer (supplied with the enzyme) with 1.5 mM $MgCl_2$, 20 pmoles each of primers (*PMI*-1:5'-ACA GCC ACT CTC CAT TCA-3'; *PMI*-2: 5' GTT TGC CAT CAC TTC CAG-3'), 200 µM each of dNTPs, and 1 unit of Taq DNA polymerase enzyme overlaid with 50 µl of mineral oil. The reaction was carried out using MJ-Research PTC - 100 thermal cycler with the following reaction conditions: initial denaturation step for 5 min at 94 °C followed by 30 cycles of 94 °C for 1 min; 60 °C for 1 min; 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. The amplification product (514 bp) was run on 1% TAE agarose gel and photo documented.

The PCR products were separated on 1% TAE agarose gel and then were blotted onto nylon membrane (Hybond N^+ Amersham Pharmacia). The 514 bp purified fragment from *PMI* gene was radio labeled with α - [^{32} P] dCTP using Random Primer Labeling kit from BRIT (India) and used as a probe. The subsequent blotting and hybridization was carried out according to Sambrook et al. (1989).

Results and Discussion

In order to determine the effects of mannose on germination and callus induction, mature seeds and mature embryo-derived callus were placed on medium supplemented with either mannose (1-4%) or sucrose (3%). None of the embryos germinated or produced callus on mannose containing CIM medium, while 95-100% seeds germinated with good root and soot system and developed callus on CIM with sucrose. Similarly, callus did not grow well on mannose-containing media, while callus grew very well (95%) on sucrose medium. While callus growth doubled (334 mg) within 2 weeks on mannose-free medium, there was less gain in growth on mannose supplemented CIM. Failure of the embryo to germinate on mannose medium is thought to be caused by energy depletion via a hexokinase-mediated pathway (Pego et al. 1999) and it is known that phosphorylation of mannose by hexokinase triggers a signaling cascade resulting in

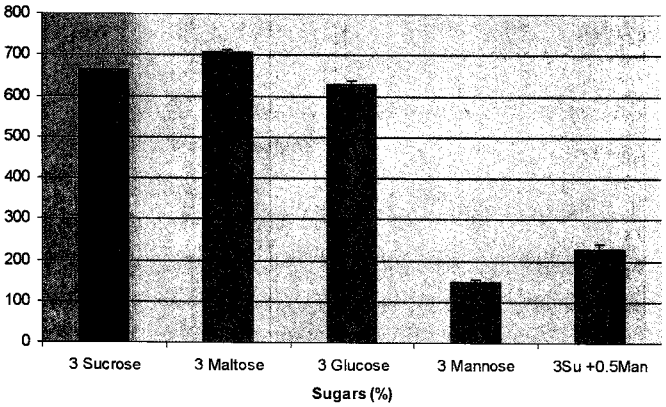

Fig. 2. Effect of different sugars on callus growth of *indica* rice cv. Pusa Basmati 1. About 150 mg initial mature embryo-derived callus was used in the experiment and final fresh weights were recorded after 4 weeks of incubation.

gene repression and energy depletion during seed germination.

Effect of sugars (sucrose, glucose, maltose, and sucrose and/or mannose) on callus was studied to check growth and whether mannose could be used as a carbon source or a selection agent (Fig. 2). Calli grew normally on medium (CIM) containing glucose, maltose and sucrose while there was no weight gain on mannose-containing medium (Fig. 2). The callus growth was almost completely arrested with mannose concentration up to 4% suggesting that the rice callus was not able to use mannose as a carbon source. As sucrose is known to suppress the inhibitory effect of mannose on growth and germination (Joersbo et al. 1998), sucrose (1.0, 0.5%) in combination with mannose (1-3%) was tested and results suggested that the proper application for selection was 3% mannose with 0.5% sucrose in the medium. Earlier studies indicated that the indirect toxic effect of mannose caused by the conversion to mannose-6-phosphate by endogenous hexokinase increases with decreasing sucrose concentration in the medium, suggesting an interaction between mannose and sucrose (Joersbo et al. 1998). The addition of sucrose, therefore, can alleviate the effect of mannose on growth and germination. In a study using fourteen different sugars suitable for use in positive, selectable marker-based transformation in chrysanthemum, Teixeira Da Silva (2004) found xylose, lactose, and cellulose as potential carbon sources for positive selection.

In the selection stage, transformed callus grew well on mannose containing medium (3%) along with 0.5% sucrose. The untransformed cells grew very slowly and eventually lost vigor after 4 weeks of culture. Selected transgenic lines were initially

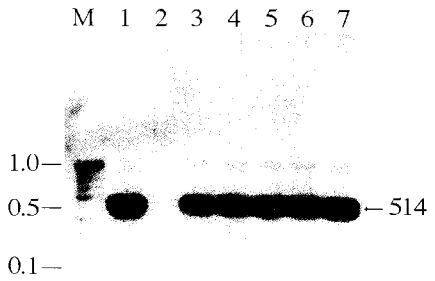

Fig. 3. PCR Southern blot analysis of HindIII digested DNA of control and transformed lines. Lane 1 is positive control, lane 2 is from control, non-transformed plants and 3-5, transformed lines. The blot was hybridized with the [32P]dCTP-labelled *PMI* gene as probe. Lane M - Lambda DNA digested with HindIII was used as a size marker (given in kb).

screened using PCR with the expected band (514 bp) while no signal was found in non-transformed controls. For checking stable transformation, PCR-positive lines were analyzed by Southern hybridization indicating that the *PMI* gene was detected in the transgenic but not in the non-transgenic lines (Fig. 3). Lucca et al. (2001) first reported the use of *PMI* as a selectable marker for the transformation of *japonica rice* cv. TP 309 with mannose selection. Selection was done with mannose concentration increased stepwise (3-5%) and regeneration was on non-mannose containing medium. Subsequently, He et al. (2004) and Zhengquan et al. (2004) also employed *PMI* gene in rice transformation of *japonica* cultivars while using mannose selection during growth and regeneration. The concentration of mannose required for efficient selection of rice (30 g/l) was ten times higher than that used for sugar beet (Joersbo et al. 1998) and almost equal to that for maize (Negrotto et al. 2000). In durum wheat transformation studies, use of *PMI* gave good selection efficiency (Gadaleta et al. 2006). Higher transformation efficiency observed in other crops like maize could be attributable to the metabolic advantage with mannose selection and absence of any toxic metabolites released from the dying non-transformed cells (often seen as necrotic spots on the callus). Lindsey and Gallois (1990) suggested that the release of toxic metabolites by non-transformed cells into the surrounding tissues might impair the regeneration and growth of the transgenic cells. Such deleterious effects of dying cells could be circumvented using mannose selection. Death under mannose accumulation in non-transformed cells is also due to transcription repression of genes associated with photosynthesis and glyoxalate cycle (Jang and Sheen 1997). In this study, callus growth was proliferative and necrotic tissues were rarely observed during selection, contrary to the dark brown or even black necrotic tissue often encountered in rice transformation experiments using hygromycin as a selection agent (Fig. 4).

PMI has been successfully applied as a selectable marker in transformation studies of many plant species. During selection with mannose, the growth of un-transformed tissue is repressed

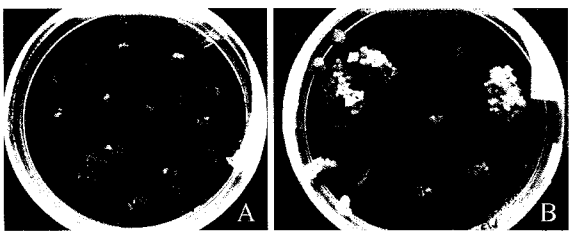

Fig. 4. Transformed rice callus growing on hygromycin (A) and mannose (B) containing selection medium.

by carbohydrate starvation and phosphate depletion, as required for ATP production. Thus in selection systems based on antibiotics or herbicides, non-transformed cells are killed, whereas the non-transformed cells in the mannose system have their growth and development arrested by carbohydrate starvation (Wang et al. 2000) but still survive (Haldrup et al. 1998). Positive selection systems are being successfully used as alternate methods to produce transformants without any antibiotic/herbicide marker gene (Suprasanna and Bapat 2005). These include *phosphomannose isomerase* (*PMI*) and *xylose isomerase* (*xylA*) genes. In a preliminary risk assessment study, Reed et al. (2001) found that

there are no safety concerns by using the *pmi* gene as a selectable marker. The authors have demonstrated that the *PMI* protein in transgenic maize was readily digested in simulated mammalian gastric and intestinal fluids, a database search revealed no significant homology of the *E. coli manA* gene product to any known toxin or allergen, and no detectable changes in glycoprotein profiles, and no significant differences in yield and nutritional composition compared to non-transformed maize. In conclusion, the results of the study indicate that *PMI* gene can be expressed in rice cells conferring the ability to use mannose as a carbohydrate source, and that the positive selection system can be used in conjunction with desirable genes to avoid using negative-selection-based marker genes.

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