Omonrice 10: 1-5 (2002)

Agrobacterium-mediated transformation of rice embryogenic suspension cells using phosphomannose isomerease gene, *pmi*, as a selectable marker

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ABSTRACT

For rice transformation, normally immature embryos or calli were used as explants for transformation. In this study, we used the embryogenic suspension cells of indica cutivars as the starting materials in the *Agrobacterium*-mediated transformation with the *pmi* gene encoding for the phosphomannose isomerase as the selectable marker. The phosphomannose isomerase system enables the transgenic cells to utilize mannose as a carbohydrate source resulting in the selection of the transgenic events on the mannose-containing medium. With this system, we were able to develop the transgenic plants from the two commercially important indica cultivars grown in the Mekong Delta, Mot Bui and MTL 250. The transgenic plants were fertile and had normal phenotype. Genomic DNA analysis confirmed the presence of the *pmi* gene in all the transformants analysed. The integration of the *pmi* gene into the genome was simple in most transformation events.

Keywords: Rice, *Agrobacterium*-mediated transformation, Mannose selection, Phosphomannose isomerase (*pmi*)

INTRODUCTION

Rice is one of the major cereal crops with great potential for biotechnology advances. Several important traits for disease resistance, drought tolerance, enhanced nutritional values have been engineered into rice in recent years. Concerning to transformation procedures of rice (*Oryza sativa* L.), so far immature embryos or calli have been used in the particle bombardment or *Agrobacterium*-mediated methods. The selection systems normally were based on either using antibiotics or herbicides as selection agents (Christou 1997, Hiei et al. 1997). The use of these selective agents has caused a common public concern because of inadequate knowledge of the agents' impact on environment and human health. To overcome this limitation, more recently, a selectable marker system based on mannose selection by using *pmi*- a gene derived from *Escherichia coli* encoding for phosphomannose isomerase (Miles and Guest 1984) was tested. This selection system was applied for the transformation of the japonica rice cv. Taipei 309 (Lucca et al. 2001) but not yet for the indica rice, the most commercially important crop in many developing countries.

The present study describes an efficient protocol for *Agrobacterium*-mediated transformation of embryogenic suspension cells of the two indica rice cultivars (cv. Mot Bui and MTL 250) using the mannose selection system. The transgenic plants developed were fertile and showed normal phenotype. Simple integration of the *pmi* gene with 1-2 copies into the rice genome was predominant.

MATERIALS AND METHODS

Bacterial strain and plasmid construction

To construct the vector pManCa, the hygromycin phosphotransferase gene of pCAMBIA 1301 (CAMBIA, Canberra, Australia) was replaced by the phosphomannose isomerase (*pmi*). For this purpose, the *pmi* gene was isolated from pCaCar (Hoa et al. 2002) by *Xho*I digestion

and cloned into the corresponding sites under the control of the constitutive CaMV 35S promoter. The binary vector was transformed into the competent cells of *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al. 1984). A single colony of *Agrobacterium* was incubated overnight in 3ml liquid YEB medium with 50 mgl^{-1} kanamycin on a shaker (240 rpm) at 28^0C. An aliquot of the bacterial suspension (250 µl) was transferred to 50 ml fresh medium and cultured for 18-20 hr to an OD_{600} 0.5-1.0. The bacteria were centrifuged at 6,000 rpm 4^0C for 10 min. The bacterial pellet was resuspended in liquid MS plasmolysis medium containing 100 g l^{-1} sucrose, 2 mg l^{-1} 2,4-D, pH 5.5 supplemented with 200 mM acetosyringone. The *Agrobacterium* culture was grown for 2-3 h at 28^0C (200 rpm) for preinduction before inoculation of the suspensions.

Initiation and maintenance of embryogenic cell suspensions

Mature dehusked seeds of the cultivars, Mot Bui and MTL 250 were surface sterilized with 70% (vol/vol) ethanol for few minutes and rinsed 2 times in sterile distilled water then in 6% calcium hypochloride for 1 h. After 3 washes with sterile distilled water, the immature embryos were isolated and cultured on MSCI medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose, 1g l^{-1} casein hydrolysate, 6 g l^{-1} agarose and 2 mg l^{-1} 2,4-diclorophenoxyacetic acid (2,4-D), pH 5.8 for 5 to 7 days. Callus was transferred to fresh MSCI medium and cultured for 1 to 2 weeks. Seven or eight friable embryogenic calli were placed into a 250-ml culture vessel containing 40 ml of N6 medium [N6 Salts and vitamins (Chu et al. 1975) supplemented with 30 g l^{-1} sucrose, 2 mg l^{-1} 2,4-D, 1 g l^{-1} tryptophan, 1 g l^{-1} proline, pH 5.8]. The cultures were maintained in dark at 28^0C on a rotary shaker at 220 rpm and subcultured at three-day intervals. Rapidly dividing, friable calli were selected and transferred 2 ml of fine callus suspension into 40 ml N6 medium and subcultured at three-day intervals. After 4 weeks, the cultures were sieved through a metallic net (ø 500µm) to obtain the fraction consisting of embryogenic units ranging in size from 250-500µm.

Transformation

Aliquots of 2ml packaged cell volume (PCV) from embryogenic suspension cells were used for inoculation with *Agrobacterium* and transferred to jars containing 20-25 ml of bacterial suspension. After 30 min, the bacterial suspension was removed and the inoculated tissues were spread onto sterile filter paper (ø 9cm) then transferred to Petri dishes with solidified MSCo medium (Aldermita and Hodges 1996) containing 200 mM acetosyringone and co-cultivated at 25^0C for 3-4 days.

Selection and regeneration of transgenic plants

The infected tissues were transferred to MS liquid medium supplemented with 250 mgl^{-1} cefotaxim and cultured for 3 days on a shaker at 150 rpm. The cultures were transferred into the same medium and cultured for 3-4 days. Selection was done every 2 weeks on MS liquid medium containing 30 g l^{-1} sucrose and 25 g l^{-1} mannose [D(+)-mannose 99%, Heros Organics, Geel, Belgium] with 250 mgl^{-1} cefotaxim for the 1st selection and 15 g l^{-1} sucrose plus 25 g l^{-1} mannose for the 2nd selection with subcultured at three-day intervals. After 4 weeks the transformed suspensions were transferred to solid MS medium with 35 g l^{-1} mannose and 5 g l^{-1} sucrose to select mannose resistant callus. The mannose resistant clones were transferred to MSCI without mannose to induce embryo development. The procedures for regeneration and rooting reported by Aldermita and Hodges (1996) were followed. Mannose resistant rice plants were transferred to soil and grown in the green-house at 29^0C (day), 21^0C (night) and 80% relative humidity.

Southern blot analysis of the regenerated plants

Genomic DNA was isolated from rice leaves following the method of McCouch et al. (1988). Ten micrograms of genomic DNA were digested with *Xho*I to detect *pmi* and with *Bam*HI for copy number analysis. Southern blot analyses were carried out following standard protocols (Sambrook et al.

1989). DIG-labelled (Boeheringer, Rotkreuz, Switzerland) of *pmi* fragment was used as probe. Hybridization, washing and detection were performed following the procedures as given in Burkhardt et al. 1997).

RESULTS AND DISCUSSION

The two rice suspension cell lines, Mot Bui and MTL 250, were used in the transformation experiments. The vector pManCa (Fig. 1) was used throughout in the present study in the *Agrobacterium*-mediated transformation. The use of *pmi* gene as the selectable marker allowed the selection of the transgenic plant tissues on the mannose-containing medium. The *pmi*-expressing cells were able to metabolize mannose into a usable source of carbon resulting in a normal growth on mannose-containing media, while the untransformed tissues either stop growing or die due to starvation (Hansen and Wright 1999). In our study, the selection of the transgenic cells was carried out on mannose-containing medium for 6-7 weeks. A total of 6 transformation experiments were performed (three for Mot Bui and three for MTL 250). The results were pooled and presented in Table 1. From a volume of 6µl of packaged cell of each cultivar, we selected 12 and 15 independent transgenic plants confirmed by Southern blot analysis for Mot Bui and MTL 250, respectively. Almost the transgenic plants were fertile and expressed their normal phenotype. Only 1 of 12 lines of Mot Bui and 2 of 15 lines of MTL 250 were completely sterile.

Molecular analysis on the DNA isolated from mannose resistant lines digested with *Xho*I using *pmi* as probe confirmed the stable integration of the transgene into the nuclear genome. No signal was detected in the DNA from untransformed plants used as negative controls (Fig. 2). Simple integration pattern of the transgene was predominant as the copy number of the integrated transgene varied from one to three. Most of the transgenic lines had one or two copies and transgene recombination did not occur (Fig. 2). These features made the *Agrobacterium*-mediated transformation method more advantageous than other methods.

For selection procedures, two months after transformation, mannose resistant colonies were recovered. The concentration of mannose during the selection was stepwise increased. It was 25 g l^{-1} at the beginning of the selection and increasing to 35 g l^{-1} for the last round of selection. Lucca et al. (2001) used high concentration of mannose during the periods of selection with 50 g l^{-1} for the third selection. The mannose concentration required for efficient selection of japonica and indica rice is higher than that reported for wheat and maize (Wright et al. 2001) and sugar beet (Joersbo et al. 1998). In contrast, the concentration of sucrose was stepwise decreased to balance the osmotic pressure of the medium and to further arrest the growth of the untransformed cells. We found that including 5 g l^{-1} sucrose in the third selection allowed escapes. Wright et al. (2001) also reported that adding small amounts of sucrose during the later stages of selection also allowed escapes. In this study, we observed that for the cultivar MTL 250, using cell suspension as starting plant material for transformation, we obtained lower percentage of escape as compared to using callus (data not shown). But to confirm this advantage of using cell suspension as explants in rice transformation more experiments should be performed. The use of suspension cells help overcoming the difficulty in growing plants to obtain immature embryos for transformation.

In conclusion, we have demonstrated that *Agrobacterium*-mediated transformation of indica rice (cell suspension) using *pmi* as the selectable marker along with the established mannose selection procedure was efficient for producing the transgenic plants. This is a significant approach in rice transformation towards the development of transgenic lines amenable to deregulation in the near future because no antibiotics or antiherbicides were used as selection agents.

Table 1. Frequency of *Agrobacterium*-mediated transformation of rice suspension cells

Cell lines	Packaged cell volume (µl)	No. of resistant cell recovered	No. of transgenic plants (Southern blot $^{+}$)
Mot Bui	6	16	12
MTL 250	6	22	15
Total	12	38	27

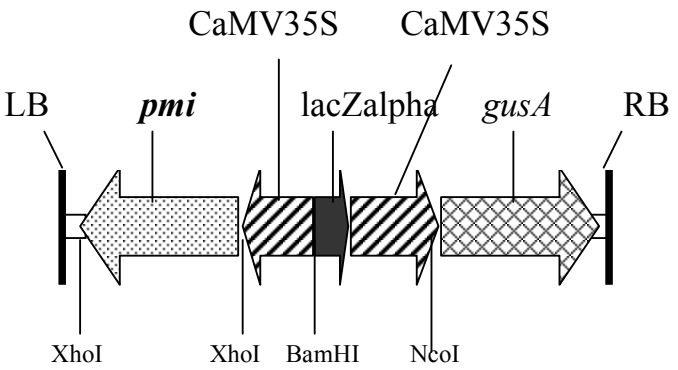

Fig. 1. Diagram of vector pManCa containing the *pmi* gene

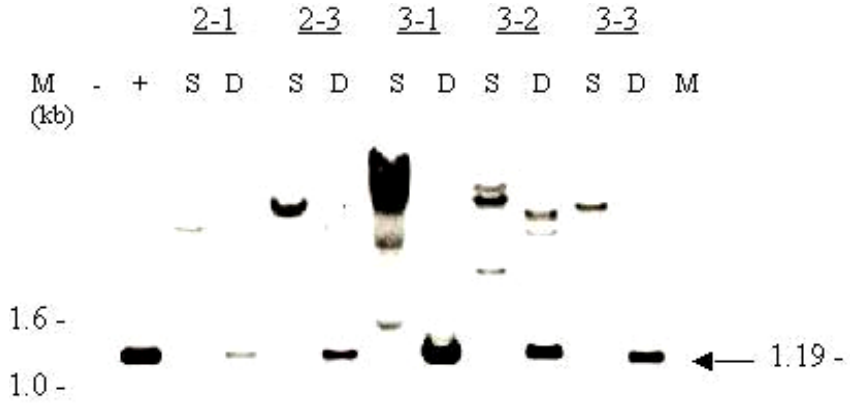

Fig. 2. Southern blot analysis: Genomic DNA of T0 plants (Mot Bui) digested with *Bam*HI as a single cutter (S) and with *Xho*I as double cutters (D). The blot was hybridized with the DIG-labelled *pmi* gene as probe.The expected transgene band size (1,190bp) is given with an arrow containing the *pmi* gene.
-: untransformed, +: Plasmid DNA.

Acknowledgments

The authors would like to thank the Rockefeller Foundation for financial support and Dr. R.A. Jefferson, CAMBIA, Canberra, Australia, for kindly providing pCAMBIA 1301.

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SUMMARY IN VIETNAMESE

Chuyển nạp gen bằng *Agrobacterium* qua tế bào huyền phù lúa, sử dụng gen chọn lọc phosphomannose isomerease, *pmi*

Trong chuyển nạp gen ở lúa, thông thường phôi non hoặc mô sẹo được sử dụng là vật liệu khởi đầu trong chuyển nạp gen. Trong nghiên cứu này, chúng tôi sử dụng tế bào huyền phù của giống lúa indica làm vật liệu khởi đầu và dùng gene tạo phosphomannose isomerease, *pmi* là gen chọn lọc. Hệ thống chọn lọc sử sụng gen *pmi* tạo các tế bào được chuyển nạp gen sử dụng mannose là nguồn carbohydrate, nhờ vậy có thể chọn lọc các tế bào được chuyển nạp trong môi trường cấy mô có thêm mannose. Sử dụng hệ thống này, chúng tôi đã thành công trong tạo ra các dòng lúa chuyển nạp gen từ hai giống lúa indica quan trọng là Một Bụi va MTL250. Các cây chuyển nạp gen cho hạt lúa hữu thụ và dạng hình nông học bình thường. Phân tích DNA khẳng định gen *pmi* đã được chuyển nạp vào hệ gen cây lúa.