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USING MANNOSE AS A POSITIVE SELECTION OF TRANSFORMED *CARICA PAPAYA* L. VAR 'EKSOTIKA'

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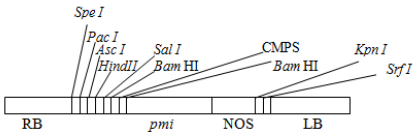
Graphical abstract



Abstract

The main objective of this study is to develop marker-free transgenic papaya plants via positive selection using phosphomannose isomerase (*pmi*) gene. Phosphomannose isomerase (PMI) is an enzyme that converts mannose-6-phosphate to fructose-6-phosphate, a glycolysis intermediate that supports the growth of plant cells. To establish a marker-free positive selection system using this PMI, the effect of mannose on the growth and development of embryogenic 'Eksotika' papaya callus was evaluated. One-month old embryogenic calli were cultured on Murashige and Skoog (MS) medium in which 60 g/L sucrose in the original recipe was replaced with different concentrations of mannose and sucrose. Mannose was supplied as the sole carbon source or in combination with sucrose at 0, 5, 10, 15, 20, 25 or 30 g/L. Embryogenic calli cultured on medium supplemented with a ratio of 0:60 g/L mannose: sucrose was used as a control. The results after six sub-cultures showed that most of the embryogenic calli transferred on media containing only mannose turned brown. Higher concentrations of mannose resulted in higher percentage of brown calli (dead). Mannose at 30 g/L mannose was found to be effective for screening transformed embryogenic calli. Evaluation of papaya transformation efficiency using this positive selection system was pursued using 650 one-month-old embryogenic calli *Agrobacterium*-transformed with pNOV2819 harboring the *pmi* gene. Only transformed cells are capable of utilizing mannose as a carbon source to grow. After five months on mannose selection, all 67 putative transformants obtained were PCR-positives for the *pmi* gene.

Keywords: 'Eksotika' papaya, positive selection, phosphomannose isomerase (*pmi*), *Agrobacterium*-mediated transformation

Abstrak

Objektif utama kajian ini adalah untuk membangunkan pokok betik transgenik penanda percuma melalui pemilihan positif menggunakan phosphomannose isomerase (*pmi*). Phosphomannose isomerase (PMI) merupakan enzim yang menukarkan mannosa-6-fosfat kepada fruktosa-6-fosfat, pengantaraan glikolisis yang menyokong pertumbukan sel-sel tumbuhan. Untuk mewujudkan satu sistem pemilihan positif percuma menggunakan *pmi* ini, kesan mannosa kepada pertumbuhan dan pembentukan embriogenik kalus betik 'Eksotika' dinilai. Kalus embriogenik yang berusia satu bulan telah dikulturkan pada Murashige dan Skoog (MS) medium dimana 60 g/L sukrosa dalam resepi asal telah digantikan dengan kepekatan mannosa dan sukrosa yang berbeza-beza. Mannosa telah dibekalkan sebagai sumber karbon yang tunggal atau dalam kombinasi dengan sukrosa pada 0, 5, 10, 15, 20, 25 dan 30 g/L. Kalus embriogenik dikultur dalam medium yang ditambah dengan nisbah 0:60 g/L mannosa: sukrosa telah digunakan sebagai kawalan. Keputusan selepas enam kali sub-kultur telah menunjukkan bahawa kebanyakkan kalus embriogenik yang ditempatkan pada media yang hanya mempunyai mannosa bertukar koko. Kepekatan mannosa yang tinggi telah menyebabkan kadar peratusan kalus-kalus berwarna koko (mati). Kepekatan mannosa pada 30 g/L telah terbukti berkesan untuk pemilihan kalus embriogenik yang ditransfom. Penilaian kebersanan transformasi betik menggunakan sistem pemilihan positif telah diuji dengan menggunakan 650 kalus yang berusia satu bulan dengan transformasi perantaraan *Agrobacterium* berserta dengan pNOV2819 yang mengandungi gen *pmi*. Hanya sel-sel yang telah ditransformasikan boleh menggunakan mannosa sebagai sumber karbon untuk berkembang. Selepas lima bulan pemilihan mannosa, kesemua 67 putatif transformasi yang diperoleh adalah PCR-positif untuk gen *pmi*.

Kata kunci: Betik 'Eksotika', pemilihan positif, phosphomannose isomerase (*pmi*), Transformasi perantaraan *Agrobacterium*

1.0 INTRODUCTION

Papaya or *Carica papaya* L. is an important horticultural crop in Malaysia. There are two popular papaya varieties grown in Malaysia, 'Eksotika' and 'Sekaki'. The 'Eksotika' papaya is a cross between 'Subang 6' and the 'Hawaiian Sunrise Solo'. It was released by the Malaysian Agricultural Research and Development Institute (MARDI) in 1987 (Chan, 1987). The sweet taste and pleasant aroma of 'Eksotika' papaya cause it to be highly demanded in the local and export markets, such as China, Hong Kong, Singapore, the Europe and Middle Ease (Sew *et al*., 2011). Current screening of putative transformed tissues in papaya transformation relies on antibiotic resistance genes or herbicide tolerance genes. The elimination of antibiotic or herbicide resistance gene in plant transformation is being encouraged due to public concern. In order to sustain further progress in this area, an alternative selection method is desired. The use of phosphomannose isomerase (*pmi*) as a positive selection enables the identification of genetically transformed tissues without further post-selection procedures. Phosphomannose isomerase (PMI) is an enzyme that catalyzes the reversible interconversion of mannose-6-phosphate to fructose-6-phosphate. The plants cells that do not have this gene are unable to survive in medium containing mannose as a carbon source. On mannose containing medium, plants cells rapidly take up mannose and convert it to mannose-6-phosphate via the action of hexokinase. However, transformed plants cells having *pmi* will convert mannose-6-phosphate to fructose-6-phospate, which is utilized further. Plants that lack *pmi* accumulate mannose-6-phosphate, that inhibits phosphoglucose isomerase and thus block glycolysis process. According to Negrotto *et al*., (2000), the accumulation and synthesis of mannose-6-phosphate also depletes orthorphosphate that is needed for ATP production. Phosphomannose isomerase gene product has no homology with any known toxin or allergen and is readily digestible as conventional dietary protein (Privalle *et al*., 2002). The transformation efficiency by using *pmi* system has been reported by many studies to be high and sometimes consider higher compared to antibiotic or herbicide selection (Boscariol *et al*., 2003; Lucca *et al*., 2001; Wright *et al*., 2000; Joersbo *et al*., 1998). The usage of *pmi* as a selectable protein was first reported by Bojsen *et al*., (1999), who successfully expressed the *pmi* gene in potato (*Solanum tuberosum* L.), sugerbeet (*Beta vulgaris* L.), and corn (*Zea mays* L.) cultured on mannose as their carbon source. This *pmi* selection system was then patented (Bojsen *et al*., 1998, 1999) and has been used successfully on *Arabidopsis* (*Arabidopsis thaliana* L.) (Todd and Tague, 2001), cassava (*Manihot esculenta* Crantz) (Zhang *et al*., 2000), corn (Negrotto *et al*., 2000; Wang *et al*., 2000;

Wright *et al.*, 2001), rice (*Oryza sativa* L.) (He *et al.*, 2004; Datta *et al.*, 2003; Hoa *et al.*, 2003; Lucca *et al.*, 2001), sugarbeet (Joersbo *et al.*, 1998, 1999), sweet orange (*Citrus sinensis* L. Osbeck) (Boscariol *et al.*, 2003), wheat (*Triticum aestivum* L.) (Wright *et al.*, 2001), and pearl millet (O'Kennedy *et al.*, 2004). In this present study, we attempted to evaluate the effectiveness of using the *pmi*/mannose as a positive selection system for 'Eksotika' papaya calli transformed using *Agrobacterium*.

2.0 EXPERIMENTAL

2.1 Embryogenesis Callus Induction of 'Eksotika' Papaya

Embryogenic callus of papaya (*Carica papaya* L.) var 'Eksotika' cultures were initiated from immature zygotic embryos obtained from Malaysian 'Eksotika' papaya fruit of 90 days after pollination. The immature embryos were cultured on the callus induction medium consisted of half-strength Murashige & Skoog (MS) medium (Murashige and Skoog, 1962), 50 mg/L myo-inositol, full strength MS vitamin (thiamine-HCL, pyridoxine, glycine and nicotinic acid), 60 g/L (w/v) sucrose, 45.2 µM 2,4-D, 0.14 g/L adenine hemisulfate, 400 mg/L glutamine, 250 mg/L carbenicillin, and 3.2 g/L gelrite. The pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min at 121°C and 15 psi. All cultures were incubated at 25 ±°C in the dark for 3-4 weeks for the induction of embryogenic calli.

2.2 To Study the Effects of various Mannose Concentrations on Embryogenic Callus Growth

To evaluate the effects of mannose on the growth and development of embryogenic 'Eksotika' papaya callus, one-month old embryogenic calli were cultured on the same but fresh medium for callus induction in which 60 g/L (w/v) sucrose in the original method was replaced with different concentrations of mannose supplied as the sole carbon source, or in combination with sucrose at 0, 5, 10, 15, 20 or 30 g/L. The calli were sub-cultured onto fresh media monthly. Visual observation on the calli growth was recorded. The embryogenic calli cultured on medium supplemented with 0:60 g/L (w/v) mannose: sucrose was used as a control.

2.3 Agrobacterium-mediated Transformation

The plasmid pNOV2819 harboring the *pmi* gene (Figure 1, and designated as pNOV2819:*pmi*) was introduced into *Agrobacterium tumefaciens* strain LBA 4404. This *Agrobacterium* strain harboring the *pmi* was inoculated in liquid Luria-Bertani (LB) medium supplemented with 50µg/ml spectinomycin and 25µg/ml rifampicin and grown at 28°C for 24 h. One-month-old embryogenic calli were transformed with pNOV2819:*pmi* using an established method for *Agrobacterium*-mediated transformation of 'Eksotika' papaya (Vilasini *et al.*, 2000). The transformed calli were selected on half-strength Murashige and Skoog (MS) basal salts medium supplemented with 30 g/L mannose supplemented with 100 mg/L cefotaxime and 150 mg/L vancomycin. Selection was carried out for a total of 5 months with one-month interval subculturings.

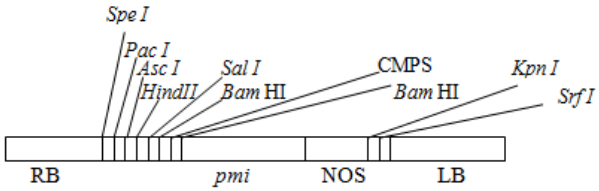


Figure 1 Plasmid map of pNOV2819 (kindly provided by Syngenta). The *pmi* gene is under the constitutive *CaMV* 35S promoter. RB: right border sequence, LB: left border sequence, *pmi*: phosphomannose isomerase

2.4 Regeneration of Putative Transformants

The mannose resistant calli that proliferated on the selection media were then transferred to De Fossard (De Fossard, 1974) maturation medium without any plant growth regulator for one month. Proliferating calli were then cultured on De Fossard regeneration medium supplemented with 0.89 µM 6-benzyladenine (BA), 1.1 µM α-naphthaleneacetic acid (NAA) and 150 mL coconut water under a light photoperiod at 26 ± 2°C, for shoot regeneration.

2.5 Molecular Analysis

The putative transgenic lines were further analyzed using PCR to verify the presence of the transgene. Genomic DNA of the papaya leaves were extracted using the Qiagen kit (Qiagen, Hilden, Germany) with a starting material of 100 mg for each sample. The PCR amplification was performed in 50µl reaction volume containing approximately 50 ng genomic DNA, 20 mM primer pairs (*pmi* forward 5'-ACAGCCACTCTCCATTCA-3' and *pmi* reverse 5'-GTTTGCCATCACTTCCAG-3'), and 1 Unit of Taq DNA polymerase. The reaction was carried out with the following PCR conditions: initial denaturation step for 3 min at 94°C followed by 35 cycles of 94°C for 1 min; 50°C for 45 sec; 72°C for 1 min, and a final elongation step at 72°C for 10 min. Twenty microliters of each amplification product was electrophoresed on 1.0% (w/v) agarose gel.

3.0 RESULTS AND DISCUSSION

3.1 Effects of Mannose on Growth and Development of Non-transformed Papaya Callus Culture

The effect of mannose on papaya calli growth was examined by culturing papaya embryogenic calli on

media containing various combination of mannose: sucrose concentrations, ranging from 0 to 30 g/L. Figure 2 shows the effect of different concentrations of mannose on growth development of embryogenic callus after six months culture with one month interval subculturings. A total of 95% of the embryogenic calli transferred on media containing only mannose turned brown and watery, and had less turgor compared to the control. Different results were observed when the calli were cultured on media containing only sucrose. The calli were healthy in all concentrations of sucrose tested, but with the highest formation of callus development in media containing 60 g/L sucrose (Figure 2b). This result indicates that sucrose plays an important role and affects the formation of somatic embryogenic callus. Abosama (2011) reported that sucrose influenced the induction of somatic embryogenesis and was one of the major factors affecting proliferation in several plant species. In 'Eksotika' papaya, it was reported that 60 g/L sucrose is the most suitable concentration for production of high frequency of somatic embryogenic callus (Vilasini *et al*., 2000). Therefore, 60 g/L sucrose was used as a control in this study. Barb *et al*. (2003) reported that although plant cells lacking the enzyme PMI are capable of converting mannose to mannose-6-phosphate, they are unable to isomerize mannose-6-phosphate to fructose-6-phosphate. As a result, the plant cells were unable to utilize the carbon source available in the tissue culture media and this eventually affected the callus growth development. Combination of mannose and sucrose also showed positive development of somatic embryos but at different frequency. However, the frequency of somatic embryos produced is not as good as calli cultured on media containing 60 g/L sucrose alone. Therefore, for selection of non-transformed tissue, media-containing a combination of mannose and sucrose were not used. As expected, the calli cultured on sugar-free medium failed to exhibit further growth, and eventually died after three months.

3.2 *Agrobacterium*-Mediated Transformation and Regeneration of Transformants

A total of 650 calli was transformed with pNOV2819: *pmi* construct by using *Agrobacterium*-mediated transformation method. The papaya embryogenic calli were co-cultivated with *Agrobacterium tumefaciens* strain LBA 4404 for three days before subjected to mannose selection. The co-cultivation medium was supplemented with 100 mg/L and 150 mg/L of cefotaxime and vancomycin, respectively, in order to eliminate the excessive growth of *Agrobacterium*. The first selection was conducted at 30 g/L mannose. After one month on selection, approximately 80% of transformed calli survived, while other turned brown (indicated as dead). The surviving calli were then subjected to subsequent screening, and survival rates of the transformed calli were further reduced and approximately 50% of the calli turned brown. After a further 5 months selection on 30 g/L mannose, the untransformed calli were eliminated, and finally 67 (10.3%) putative transformed calli were recovered. Figure 3 shows transformed 'Eksotika' papaya after 5 months on selection. The surviving putative transgenic calli were subcultured on the maturation medium without plant hormone for further development of the somatic embryos. After 1 month on this medium, the somatic embryos were transferred onto regeneration medium (supplemented with BAP and NAA) to form plantlets. Addition of coconut water in the regeneration medium helped to increase shoots formation. In this present study, 5 to 6 shoots were obtained from each individual cluster of somatic embryos. The regeneration of the transformed lines was carried out for 7 months with one-month interval of subculturings until the shoots reached approximately 4 cm.

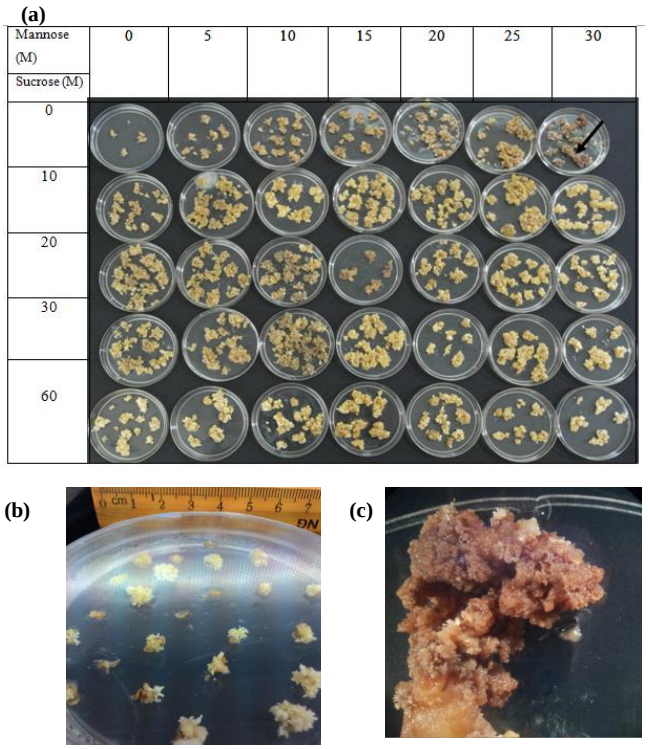


Figure 2 Effect of mannose on growth and development of embryogenic papaya calli after 5 months. a: Mannose at 30 g/L resulted in higher percentage of brown calli (dead, as indicated with arrow), b: Calli on 60 g/L sucrose, c: A close-up view of callus at 0:30 g/L sucrose:mannose

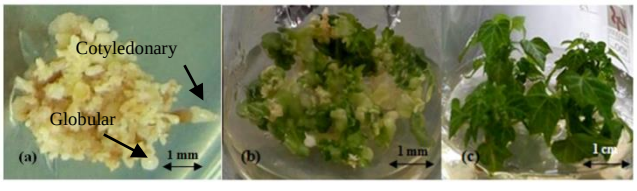


Figure 3 *Agrobacterium*-mediated transformation and regeneration of 'Eksotika' papaya. a: Selection of transformed somatic embryos (arrows indicate globular and cotyledonary stages), b: Germination of putative transformed tissues and c: Regenerated plantlets. (Scale bar = 1mm and 1cm)

3.2 PCR Analysis of Putative Transgenic Papaya Lines

Figure 4 shows the sixty-seven regenerated putative transformed lines were obtained after 5 months selection on 30 g/L mannose. To verify the presence of the transgene in the putative transformants (designated as transformed R_0 lines), PCR analysis was carried out on genomic DNA extracted from approximately 8-10 months old regenerated shoots. From a total of 67 samples analyzed, all were positive for the presence of the 514 bp *pmi* gene. Overall, the percentage of putative transgenic papaya lines obtained was 10% based on the total number of calli transformed.

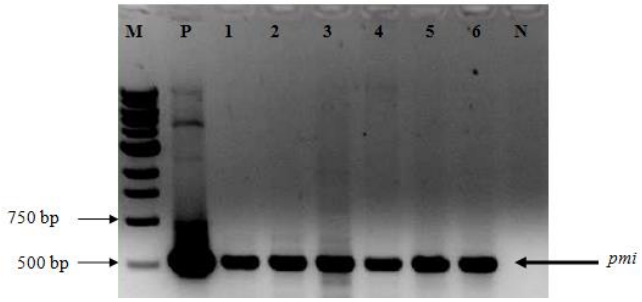


Figure 4 PCR analysis of putative transgenic lines. Lane M: 1 kb DNA ladder (Biolabs, California, USA), lane P: positive control (pNOV2819:*pmi* plasmid), lanes 1-6: transformed putative lines, and lane N: negative control (sterile distilled water). The expected size of the *pmi* gene (arrow) is 512 bp

4.0 CONCLUSION

Findings in this study revealed that the *pmi* gene could be expressed in papaya cells conferring the ability to use mannose as a carbon source for growth, and the *pmi*/mannose positive selection system can be used to avoid using negative-selection-based marker genes for future transformation of 'Eksotika' papaya. A suitable concentration of mannose to use for 'Eksotika' somatic embryos transformation is at 30 g/L.

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