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Biolistic transformation of oil palm using the phosphomannose isomerase (pmi) gene as a positive selectable marker

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The selectable marker system based on the Escherichia coli phosphomannose isomerase (pmi) gene was evaluated for the genetic transformation of oil palm. Four transformation vectors were constructed for transforming oil palm. The transformation vectors both carried the pmi gene alone or in combination with the β-glucuronidase (gusA) gene, and were driven by either the maize ubiquitin promoter (pMI11 and pMI11G) or the CaMV35S promoter (pMI3 and pMI3G). The four transformation vectors were transformed into oil palm embryogenic calli via biolistic-mediated transformation. For stable transformation experiments, bombarded oil palm embryogenic calli were selected on a medium supplemented with mannose as the only carbon source (without sucrose) one month after bombardment. Transformed embryogenic calli that survived selection on mannose were later isolated, proliferated and regenerated into whole plantlets on a regeneration medium containing mannose. The status of the regenerated transgenic plantlets was confirmed by polymerase chain reaction (PCR) and Southern hybridization. Transgene expression was detected by reverse transcription (RT)-PCR analysis. The results of this study indicate that a mannose-based selection system can be successfully used in oil palm transformation.

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1. Introduction

The oil palm (Elaeis guineensis Jacq.) is the most important commodity crop in Malaysia. It is a perennial monocotyledonous tree, and is used mainly to produce palm oil from the fleshy mesocarp of its fruits besides the lauric-rich kernel oil which is used mainly in non-food applications. Due to the importance and the endless list of products derived from the fruits and the palm, the palm oil industry will continue to be significant for the country.

A genetic engineering approach has been initiated for improving oil palm productivity and to synthesize novel high value-added products. It also aims at speeding up the genetic modification process from this long-generation and land-demanding crop. In facing the ever-changing world of agro-biotechnology, many genetic transformation methods have been developed. One such method is by biolistic which was first reported by Sanford et al. (1987) was initially chosen as a method for oil palm transformation (Parveez, 2000). It has been the most successful method for transforming monocotyledons and agronomically important crops (Potrykus, 1991). The successful regeneration of transgenic oil palm, for introducing novel traits, has relied largely on the use of negative selectable marker genes. These negative selectable marker genes routinely used in oil palm transformation are genes that confer resistance to the antibiotic hygromycin (hygromycin phosphotransferase [hpt]) and the herbicide Basta (phosphinothricin acetyltransferase [bar]) (Parveez and Christou, 1998; Abdullah et al., 2005). Besides the use of suitable selectable marker genes, the use of suitable target tissues is also important for the successful production of transgenic plants. Production of transgenic oil palm using embryogenic calli as the target tissue has been established (Parveez, 2000). Recently, Kanchanapoom et al. (2008) also reported that embryogenic calli is the most suitable target tissue for biolistic transformation of oil palm when compared to immature embryos and young seedlings, with a very high transformation efficiency obtained. In contrast, immature embryos are the preferred target tissue for oil palm transformation by another laboratory (Abdullah et al., 2005).

The elimination of antibiotic or herbicide resistance gene usage in plant transformation for genetically modified organisms is being encouraged due to public concern. In response to this, alternative selection systems for the recovery of transgenic plants have been developed using positive selectable markers (Haldrup et al., 1998; Joersbo et al., 1998; Hansen and Wright, 1999). Positive selection systems using mannose (Joersbo et al., 1998, 1999), xylose (Haldrup et al., 1998) or deoxyglucose (Kunze et al., 2001), have been shown to be more efficient selection agents in a number of crops than methods based on antibiotic selection (Zhang et al., 2000). Among the available genes for positive selection, the pmi...
gene encoding phosphomannose isomerase (PMI) from Escherichia coli (Miles and Guest, 1984) was discovered as a novel selectable marker and suitable for plant transformation (Joersbo and Okkels, 1996; Bojen et al., 1998). PMI is a necessary enzyme for mannose metabolism in vascular plants. In the presence of mannose in transformed cells, PMI converts mannose-6-phosphate into fructose-6-phosphate. The product can be immediately incorporated into the plant metabolic pathway (Reed et al., 2001). Thus, the mannose can be used as the sole source of carbohydrate by the transformed cells. Mannose cannot be usually metabolized by non-transformed cells and is converted into mannose-6-phosphate by endogenous hexokinase. Therefore, when mannose is added to the culture medium, it can minimize plant growth due to mannose-6-phosphate accumulation. Moreover, in terms of safety, the PMI protein is readily digestible in a simulated gastric environment which showed no adverse effects in an acute mouse oral toxicity experiment, indicating low allergic potential (Privalle, 2002).

The PMI/mannose selection system has been successfully used for the regeneration of transgenic plants from several economically important crops such as sugar beet (Joersbo et al., 1998), wheat (Wright et al., 2001), maize (Wright et al., 2001), rice (Luca et al., 2001), pearl millet (O’Kennedy et al., 2004), bentgrass (Fu et al., 2005), some woody species such as sweet orange (Boscariol et al., 2003), almond (Ramesh et al., 2006) and also the model plant Arabidopsis (Todd and Tague, 2001). Thus, in an effort to further improve the efficiency of oil palm genetic transformation, the use of positive selectable markers was proposed for evaluation. Therefore, the objective of this study was to produce transgenic oil palm by using mannose as a selectable agent and the pmi gene, driven by the CaMV 35S and Ubi1 promoters. The two constitutive promoters, CaMV35S and Ubi1 promoters, were chosen to drive the pmi gene in this study because they have been proven to be effective in oil palm transformation studies (Chowdhury et al., 1997). The CaMV35S promoter used in a number of different plant species has been shown to be constitutive and highly active especially in dicot plants, whereas the Ubi1 promoter has been characterized as a suitable promoter for monocots (Christensen and Quail, 1996).

2. Materials and methods

2.1. Construction of pMI3, pMI11, pMI3G and pMI11G transformation vectors

All DNA manipulations were performed according to standard protocols (Sambrook and Russel, 2001). The pmi gene of E. coli strain XL-1 Blue was PCR-amplified using gene-specific primers and the resulting PCR product was cloned into the PCR2.1TOPO vector (Invitrogen). The resultant construct (PCRiiM2-68) was verified by DNA sequencing. The construct was later digested with NheI to yield the 1.1 kb fragment of the pmi gene, and was gel-purified prior to ligation into the Avrl site of pMB3 (CaMV35S-Nos in Bluescript SK) and pMB11 (Ubi1-Nos in Bluescript SK) (Masani et al., 2008). The positive clones were confirmed by BamHI digestion. The sense orientation of the pmi gene was confirmed by PCR amplification using PMI forward (5′-CCGGCTAGCCTTGGAAAGATCTTACTACAG-3′) and Nos reverse (5′-GGACCTAGTGGCGATCTGATACATGATGA-3′) primers. The amplification was performed as follows: 95 °C for 5 min to denature the DNA template and then 30 times of 1 min denaturing at 95 °C, 1 min annealing at 60 °C and 3 min elongating at 72 °C. The reaction was repeated 30 times, with a final 5 min of elongation at 72 °C. The two transformation vectors, designated as pMI3 and pMI11, were confirmed by digestion with BamHI, BglII, EcoRI and HindIII restriction enzymes. The plasmids pMI3 and pMI11 contain the pmi gene, under the control of the CaMV35S and Ubi1 promoters, respectively. The two vectors, pMI3 and pMI11, were also further modified by the introduction of the gusA gene driven by the CaMV35S and Ubi1 promoters, respectively. The reporter gene was used to monitor gene transfer transiently. The DNA fragment of CaMV35S-gusa-Nos was excised from pBl221 by digestion with HindIII and EcoRI, gel-purified and ligated to the HindIII and EcoRI sites of pMI3 to create pMI3G. Similarly, the Ubi1-gusa-Nos fragment was excised from pAH27 (Christensen et al., 1992) by HindIII digestion, gel-purified and ligated to the HindIII site of pMI11 to create pMI11G. All four vectors constructed for expressing the pmi gene designated as pMI3, pMI3G, pMI11 and pMI11G were sequenced to confirm the integrity of the plasmid DNA (GenBank accession no: M15380). Based on the complete sequence of each vector, a restriction map was drawn using the Vector NTI software (Invitrogen) as shown in Fig. 1.

2.2. Plant materials and culture conditions

Oil palm embryogenic calli cultures were derived from an E. guineensis var. Tenera liquid suspension culture initiated from unopened immature leaves. This was kindly provided by Dr. Ahmad Tarmizi from Tissue Culture Group, MPOB. Approximately 0.5 g fresh weights of embryogenic calli were used for the bombardment experiments. The embryogenic calli were cultured on agar-solidified medium containing MS macro and micronutrients (Murashige and Skoog, 1962) supplemented with 1 mg/l naphthalene acetic acid (NAA) and 30 g/l sucrose (Parveez and Christou, 1998). The medium was adjusted to pH 5.7 with NaOH, and autoclaved at 121 °C for 15 min. All tissues were incubated in the dark at 28 °C.

2.3. Biolistic transformation of oil palm embryogenic calli with PMI transformation vectors

Optimization of the parameters involved in the transformation of oil palm has been achieved previously (Parveez et al., 1997, 1998). In the bombardment process, the plasmid DNA was extracted using the Maxi Preparation Kit (Qiagen). The DNA was precipitated and resuspended in 1 ml TE buffer to a final concentration of 300–500 μg/μl. Microcarrier preparation was carried out according to the Biostic PDS-1000/He Particle Delivery System Instruction Manual (BioRad). Five micrograms of plasmid DNA solution, 50 μl of CaCl2 (2.5 M) and 20 μl spermidine (0.1 M, free-base form) were added sequentially to the 50 μl gold particle suspension. The mixture was vortexed for 3 min, spun for 10 s at 10,000 rpm, and the supernatant discarded. The pellet was washed with 250 μl of absolute ethanol. The final pellet was resuspended in 60 μl of absolute ethanol. Six microlitres of the solution were loaded onto the centre of the macrocarrier and was air-dried. Bombardments were carried out once at the following conditions: 1100 psi rupture disc pressure, 6 mm rupture disc to macrocarrier distance, 11 mm macrocarrier to stopping plate distance, 75 mm stopping plate to target tissue distance, and 67.5 mmHg vacuum pressure (Parveez et al., 1997, 1998).

A total of 30 plates of embryogenic calli were bombarded with each of the following vectors: pMI3, pMI3G, pMI11 and pMI11G. Then, the bombarded embryogenic calli were either subjected to transient gusA gene expression or selected for regenerating stably transformed plants.

2.4. Selection and regeneration

One month after gene delivery, the bombarded embryogenic calli were transferred onto embryogenic calli medium containing 30 g/l mannose as the selection agent. Sucrose was not added to the above medium, and mannose was used to serve as the only carbohydrate source in the cultures. The bombarded embryogenic calli were grown under 16/8 h light conditions at 28 °C. The embryogenic calli were subcultured every four weeks onto fresh medium under selection.
After a period of six to twelve months, the initial regenerated shoots were placed onto shoot-inducing medium and subsequently onto root-inducing medium (Table 1).

2.5. Transient GUS histochemical assay

Expression of the gusA gene was assayed using a modified protocol of Jefferson (Jefferson, 1989). Two pmi gene transformation vectors, pMI3G and pMI11G, carrying the gusA gene were bombarded into oil palm embryogenic calli, and the experiment was carried out in 5 replicates. The bombarded calli were incubated in the dark at 28 °C for 48 h. The calli were later stained overnight (20 h) at 37 °C in GUS buffer (0.2 M sodium phosphate buffer (pH 7.0), 0.5 mM K4[Fe(CN)6]; 0.5 mM K3Fe(CN)6), X-GlucA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; 0.5 mg/ml) dissolved in dimethyl sulfoxide (DMSO). Blue spots were scored optically using a Nikon UFX-DX system.

2.6. DNA isolation and polymerase chain reaction

Genomic DNA isolation was carried out using the modified (Doyle and Doyle, 1987) method. For PCR analysis, 600 ng of DNA samples, from both transformed and untransformed oil palm, were used for
each reaction, while 100 ng of plasmid DNA was used as the positive control. Each PCR reaction was carried out in 25 μl reaction mixture containing 2.5 μl 10 × PCR buffer (Promega), 3.0 μl 25 mM MgCl₂ (Promega), 0.5 μl 10 mM dNTP (Promega), 1.0 μl 10 μM appropriate primers; forward and reverse and 0.2 μl 5 U/μl Taq polymerase (Promega), and an appropriate amount of template DNA. Total volume was made up with sterile distilled water. PCR was carried out using the PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). A 1.1 kb band was expected to be amplified using random priming by a Random Primer 12 DNA labelling kit and plantlets in six to twelve months. Concurrently, the bombarded embryogenic calli manage to proliferate and regenerate normal shoots would eventually lead to somatic embryo formation and maturation, inhibitory concentration of mannose replacing sucrose as the carbohydrate source for stopping the growth of embryogenic calli through starvation. Results on the effect of mannose selection experiment are summarized in the form of ANOVA (Table 2), obtained on seven different combinations of mannose and sucrose medium at 0, 5, 10, 15, 20, 25 or 30 g/l maintaining total sugars at 30 g/l. The data presented that fresh weight of embryogenic calli grown on 30 g/l sucrose was significantly different to embryogenic calli grown on 30 g/l mannose. As interest, this 30 g/l mannose concentration was chosen after determining the minimal inhibitory concentration of mannose replacing sucrose as the carbohydrate source for stopping the growth of embryogenic calli through starvation.

3. Results and discussion

3.1. Selection and regeneration of transformed embryogenic calli

Bombarded tissues were first cultured on embryogenic calli medium in the absence of a selection agent for roughly one month. As a control, the untransformed embryogenic calli were subcultured on normal embryogenic calli medium supplemented with 30 g/l sucrose. The embryogenic calli proliferated normally and the process would eventually lead to somatic embryo formation and maturation, shoot regeneration, rooting and finally the recovery of new viable plantlets in six to twelve months. Concurrently, the bombarded embryogenic calli and untransformed embryogenic calli were subjected to selection on embryogenic calli medium supplemented with 30 g/l mannose to fully supplement the 30 g/l sucrose. This 30 g/l mannose concentration was chosen after determining the minimal inhibitory concentration of mannose replacing sucrose as the carbohydrate source for stopping the growth of embryogenic calli through starvation. Results on the effect of mannose selection experiment are summarized in the form of ANOVA (Table 2), obtained on seven different combinations of mannose and sucrose medium at 0, 5, 10, 15, 20, 25 or 30 g/l maintaining total sugars at 30 g/l. The data presented that fresh weight of embryogenic calli grown on 30 g/l mannose was significantly different to embryogenic calli grown on 30 g/l sucrose. It was observed that none of the untransformed embryogenic calli manage to proliferate and regenerate normal shoots on medium supplemented with 30 g/l mannose. Upon transfer to fresh medium containing the selection agent, i.e. 30 g/l mannose, the untransformed embryogenic calli grew very slowly, become brown and eventually showed reduced overall tissue vigour (Fig. 2a). On the other hand, when the transformed embryogenic calli were exposed to fresh embryogenic calli medium containing for semi-quantitative RT-PCR, total RNA was treated with DNase to prevent amplification of genomic DNA. RNA was reverse transcribed to cDNA by using High Capacity cDNA Archive Kit (Applied Biosystems). The cDNA was directly used for the PCR reaction. An amount of 2 μg of first strand cDNA were amplified in the presence of the 5 μl of gene specific primers for PMI, together with the 18S primers; forward (5′-CAAAGCAAGCTCCTGCTCGT-3′) and reverse (5′-GGCTTCCACAACTAAAGAG-3′), used an internal control. Complete reaction mixtures were aliquoted and placed into a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). The amplification was performed as follows: 94 °C for 5 min to denature the DNA template and then 34 times of 1 min denaturing at 94 °C, 1 min annealing at 50 °C, and 1 min 5 s elongating at 72 °C. The reaction was repeated 34 times, with a final 5 min of elongation at 72 °C. PCR products were separated electrophoretically, and visualized by EtBr staining.

3.2. Southern blot hybridisation

Genomic DNA (30 μg) was digested with SacI restriction enzyme for 16–20 h at 37 °C, and separated by electrophoresis in 1% agarose gel with tris-borate-EDTA (TBE) buffer (Sambrook et al., 1989) and transferred onto nylon Hybond-N+ membrane (Amersham). Southern hybridisations were performed according to Church and Gilbert (Church and Gilbert, 1984) with modifications. The membranes were probed with the 1.1 kb fragment of the pmi gene labelled with [α-32P]dATP using random priming by a Random Primer 12 DNA labelling kit (New England Biolabs). Hybridisation was carried out at 55 °C in sodium phosphate buffer [0.5 M sodium phosphate, 1 mM EDTA and 7% (w/v) SDS]. The membrane was washed with 2 × SSC/0.1% (w/v) SDS for 10 min followed by 1 × SSC/0.1% SDS at 55 °C for 10 min. The membrane was exposed to an X-ray film (Kodak Photo Film) at −70 °C for 7 days.

2.9. Total RNA isolation and reverse transcription (RT)-PCR

Total RNA extraction from oil palm tissues including embryoids and plantlets was carried out according to Zeng and Yang (2002). For semi-quantitative RT-PCR, total RNA was treated with DNase to prevent amplification of genomic DNA. RNA was reverse transcribed to cDNA by using High Capacity cDNA Archive Kit (Applied Biosystems). The cDNA was directly used for the PCR reaction. An amount of 2 μg of first strand cDNA were amplified in the presence of the 5 μl of gene specific primers for PMI, together with the 18S primers; forward (5′-CAAAGCAAGCTCCTGCTCGT-3′) and reverse (5′-GGCTTCCACAACTAAAGAG-3′), used an internal control. Complete reaction mixtures were aliquoted and placed into a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). The amplification was performed as follows: 94 °C for 5 min to denature the DNA template and then 34 times of 1 min denaturing at 94 °C, 1 min annealing at 50 °C, and 1 min 5 s elongating at 72 °C. The reaction was repeated 34 times, with a final 5 min of elongation at 72 °C. PCR products were separated electrophoretically, and visualized by EtBr staining.

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Table 1

Selection media for in vitro cultures of E. guineensis at different stages.

<table>
<thead>
<tr>
<th>Media</th>
<th>Embryogenic calli</th>
<th>Shoot formation</th>
<th>Root formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS salts including vitamin (g/L)</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Myoinositol (g/L)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>NAA (μM)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Arginine (g/L)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Asparagine (g/L)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Activated charcoal (%)</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Plant agar (g/L)</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Mannose (g/L)</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Ph</td>
<td>5.8</td>
<td>5.8</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Table 2

ANOVA from different combination mannose and sucrose media of mean fresh weight embryogenic calli.

<table>
<thead>
<tr>
<th>Media</th>
<th>Mannose: Sucrose (g/L)</th>
<th>Mean of weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.30</td>
<td>154.45 ± 0.29f</td>
</tr>
<tr>
<td>2</td>
<td>5.25</td>
<td>130.51 ± 0.26d</td>
</tr>
<tr>
<td>3</td>
<td>10.20</td>
<td>101.13 ± 0.32bc</td>
</tr>
<tr>
<td>4</td>
<td>15.15</td>
<td>126.03 ± 3.70d</td>
</tr>
<tr>
<td>5</td>
<td>20.10</td>
<td>111.48 ± 4.00c</td>
</tr>
<tr>
<td>6</td>
<td>25.5</td>
<td>107.12 ± 2.90bc</td>
</tr>
<tr>
<td>7</td>
<td>30.0</td>
<td>98.46 ± 0.57f</td>
</tr>
</tbody>
</table>

Note: Treatment means with the same letter are not significantly at p=0.05 according to Duncan’s Multiple Range Test. Values represent the mean ± S. E. of five replicates for five months.
30 g/l mannose, mannose-resistant embryogenic calli colonies started to emerge normally after five to six months of selection. The pmi-transformed and mannose-selected embryogenic calli structures appeared yellow, healthy and grew vigorously indicating that the pmi-transformed calli could effectively utilize mannose as their carbon source. In oil palm, the time taken by the transformants to recover is quite long as compared to rice where mannose-resistant colonies recovered two months after transformation (Hoa and Bong, 2002).

The resistant colonies were later proliferated and regenerated into transgenic oil palm plants. The pmi-transformed embryogenic calli (Fig. 2b) were proliferated on the same medium containing mannose until the size of the colony became sufficiently big and turned into embryos (Fig. 2c). The transgenic embryos began to regenerate on the selection medium. Whitish embryos and followed by greenish polyembryogenic calli started to develop after three to five months of culture on polyembryogenic-inducing medium. After two to three months, some of these polyembryogenic cultures started to produce shoots (Fig. 2d and e). Once these shoots were big enough, they were individually isolated from the polyembryoids cultures and transferred into conical flasks or test tubes containing shoot-inducing medium for shoot elongation (Fig. 2f). After another two to three months, the elongated shoots were transferred into test tubes containing root-inducing liquid medium for further development and root initiation. After about two months in the liquid root-inducing medium, individual plantlets with good rooting systems were obtained (Fig. 2g). While Fig. 2h and i shows the complete plantlet and hardened plant in soil. No significant difference in shoot growth and morphology was observed between the four constructs used in this study (pMI3, pMI11, pMI3G and pMI11G). The results suggest that the deleterious effects of dying cells on the regeneration of transgenic oil palm were to a large extent avoided when using mannose as the selection agent. It was previously reported that, regardless of the transformation method, whether via biolistic, protoplasts or Agrobacterium, the PMI selection system has proven to be efficient for maize and wheat (Wright et al., 2001). It has also been demonstrated to work well in other monocots such as rice and barley (Wang et al., 2000; Ding et al., 2006). The mannose-based selection system has also been demonstrated to be successfully used in the transformation of several dicotyledonous species such as sugar beet (Joersbo et al., 1998), tomato (Bríza et al., 2008), sorghum (Gurel et al., 2009) and potato (Bríza et al., 2008).

Joersbo et al. (1998) has previously reported that mannose is a better carbohydrate source than sucrose because it allows for faster growth rates in transgenic sugar beet. Its effect has been correlated to pmi gene activity in transgenic plants. Saccharide composition and phosphate content in the selection medium have been shown to have an impact on the effect of mannose selection (Joersbo et al., 1999).
Compared to other sugars such as glucose and fructose, sucrose resulted in a higher transformation frequency in sugar beet. It was also reported that the addition of phosphate had a strong positive effect on the transformation frequencies. Interestingly, it was reported in Carrizo citrange and Pineapple sweet orange that transformation responses were opposite for the mannose and sucrose combinations (Ballester et al., 2008). Carrizo citrange was shown to have 30% transformation efficiency on medium containing 12.5 g/l mannose: sucrose. In contrast, no transformation was observed when only mannose was added as the carbon source in the selection medium. On the other hand, Pineapple sweet orange was easily transformed using both sugar combinations. A higher percentage of transgenic shoots was obtained after selection on medium containing only 15 g/l mannose as compared to selection on a 12.5 g/l mannose:sucrose combination. In lettuce, it was reported that higher transformation efficiency was obtained after selection on 20:20 g/l mannose:sucrose (Bitza et al., 2010). No shoots were produced on medium without sucrose or with low sucrose (10 g/l). Sucrose protects the plants against mannose which can inhibit the organogenesis of lettuce cotyledon leaves. Higher protection was obtained on an intermediate content of sucrose (20 g/l). Similar results were reported for tomato where the addition of sucrose to the selection medium reduced the inhibitory effect of mannose on shoot formation (Sigareva et al., 2004). Therefore, it is believed that further modification of the mannose selection protocol may improve the transformation frequencies in oil palm.

It is demonstrated in Table 3 that the number of embryoids and transgenic plant events regenerated from the Ubi1 promoter-based vector was not much different from the number from the CaMV35S-based promoter. It could be suggested that based on number of embryoids and transgenic plants regeneration the Ubi1 promoter did not show any difference in the efficiency of regenerating transgenic oil palm using pmi gene as compared to the CaMV35S promoter. This is interesting and needs further investigation to determine why the Ubi1 promoter which originated from a monocot plant, maize, does not show any advantage over the dicot-favorable promoter, CaMV35S, in this monocot plant in term of transgenic plants production using pmi gene. In contrast, Chowdhury et al. (1997) and Masura et al. (2010) have shown that the Ubi1 promoter was better than the CaMV35S in driving the transient expression of the gusA reporter gene in oil palm embryogenic calli and embryoids. The observation that there was no correlation between promoter strength and efficiency of transgenic plants regeneration demonstrated in oil palm using the pmi gene is in agreement with results reported by Joersbo et al. (2000) for sugar beet transformation. The evidence for a beneficial effect of a strong promoter driving the selectable marker gene was not obvious in this study. It has been reported that in Arabidopsis thaliana a relatively weak promoter may be superior in terms of transformation efficiency as compared to a stronger promoter.

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>pmi gene driven by the CaMV35S promoter (pMI3 &amp; pMI3G)</th>
<th>pmi gene driven by the Ubi1 promoter (pMI11 &amp; pMI11G)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of sample regenerates and tested</td>
<td>No. of samples with positive results</td>
</tr>
<tr>
<td>PCR</td>
<td>RT-PCR</td>
<td>PCR</td>
</tr>
<tr>
<td>Embryo</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Plantlet</td>
<td>8</td>
<td>–</td>
</tr>
</tbody>
</table>

- Not done due to unavailability of samples.

**Fig. 3.** Comparison of transient histochemical gusA gene expression in (from left) (a) oil palm embryogenic callus and (b) embryoid bombarded with plasmids carrying the gusA gene driven by different promoters: (i) pMI3G (CaMV35S) and (ii) pMI11G (Ubi1), and (iii) bombarded without DNA.
According to Duncan (Mengiste et al., 1997), similarly, it was demonstrated that promoter strength may appear to be less important in increasing the transformation efficiency of Indica rice (Li et al., 1997).

3.2. Analysis of transformants

3.2.1. Transient GUS histochemical assay

Oil palm embryogenic calli bombarded with the pM13G and pM11G transformation vectors, carrying the pmi and gusA reporter genes, and embryogenic calli bombarded with only gold particles without DNA (as a negative control) were histochemically assayed for gusA expression by staining with GUS buffer. This was done to ensure that the constructs used were successfully transferred into the oil palm embryogenic calli. Fig. 3 shows that blue spots were detected in calli bombarded with the pM13G and pM11G constructs. No GUS activity was observed in the negative control embryogenic calli. The result clearly suggests that the blue spots observed were due to the introduced gusA reporter gene which confirmed the transgene activity. It was also observed that number of transient expression of gusA gene driven by ubiquitin promoter (pM11G) was significantly higher than the transient expression of gusA gene driven by CaMV35S promoter (pM13G) as demonstrated in Tables 4 and 5. This shows that the activity of ubiquitin promoter is superior to CaMV35S in oil palm embryogenic calli and embryos.

3.2.2. PCR analysis

Initial screening of the putative transgenic plants was conducted at a molecular level using PCR analysis. The analysis was performed to examine the presence of the pmi gene in the putative embryos and transformed plantlets. Genomic DNA was isolated from a portion of the recovered embryos and plantlets, and subjected to PCR analysis. The presence of the pmi gene was verified using the PMI-F and PMI-R primers. Fig. 4 shows the putative transformed embryos and plantlets produced amplified fragments with the expected DNA size (1.1 kb), demonstrating the presence and presumably the insertion of the pmi gene into the oil palm genome DNA. No PCR product was observed in the untransformed oil palm plants (Lane U), while the positive controls (DNA of the transformation vector, Lane P) produced a PCR product of similar size (1.1 kb). It was found that 10% of the embryos that were analyzed failed to amplify the pmi gene, and could be considered as selection escapes. It has been recommended that a prolonged period on medium containing the selection agent is more important than the actual concentrations of the selection agent in order to reduce the production of escapes (Christiansen et al., 2005). It was shown in cucumber and sugar beet that the relative concentrations of sucrose to mannose served to increase the frequency of escapes, indicating the importance of selection agent concentration (He et al., 2006; Jain et al., 2007). It was demonstrated in carnation that a very high concentration of the selection agent, 200 mg/l hygromycin, was required to produce transgenic plants without the occurrence of

![Fig. 3](image-url)

**Fig. 3.** Blue spots detected in calli bombarded with the pM13G and pM11G constructs. No GUS activity was observed in the negative control embryogenic calli. The result clearly suggests that the blue spots observed were due to the introduced gusA reporter gene which confirmed the transgene activity.

**Fig. 4.** PCR analysis of oil palm embryoids and plantlets using PMI-F and PMI-R primers to amplify the pmi gene. The expected fragment (1.1 kb) is indicated by arrows. Lane M—1 kb plus DNA ladder marker; W—water (negative control); P—pM13 (positive control); U—untransformed; 1–21—pmi-transformant (embryoids); 22–32—pmi-transformant (plantlets).

**Fig. 5.** Total RNA isolated from oil palm embryos. Each lane was loaded with 5 μg of total RNA. Intact RNA (28S and 18S) was observed. Lanes 1–11—pmi-transformant.

**Fig. 6.** RT-PCR for the detection of a 530 bp 18S fragment. Lane M—1 kb plus DNA ladder marker; Lanes 1–11—pmi-transformant.

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**Table 4**

ANOVA from two different plasmid driving gusA gene on embryogenic calli.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mean number of transient expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pM11G</td>
<td>32 ± 6.8*</td>
</tr>
<tr>
<td>pM13G</td>
<td>12.67 ± 4.46*</td>
</tr>
</tbody>
</table>

*Note: Treatment means with the same letter are not significantly at p = 0.05 according to Duncan’s Multiple Range Test. Values represent the mean ± S. E. of five replicates for five months.*

**Table 5**

ANOVA from two different plasmid driving gusA gene on embryos.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mean number of transient expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pM11G</td>
<td>758 ± 85.8*</td>
</tr>
<tr>
<td>pM13G</td>
<td>326 ± 84.36*</td>
</tr>
</tbody>
</table>

*Note: Treatment means with the same letter are not significantly at p = 0.05 according to Duncan’s Multiple Range Test. Values represent the mean ± S. E. of five replicates for five months.*
escapes (Kinouchi et al., 2006). In potato, transformation efficiency was induced by a prolonged culture period of the transformants on a medium containing 5 g/l mannose as well as by an increase in mannose concentration from 1 to 2.5, 5 and 10 g/l (Ding et al., 2006). In rice, it was reported that addition of 5 g/l sucrose in the selection stage resulted in the occurrence of escapes (Hoa and Bong, 2002). In maize, it was also reported that adding a small amount of sucrose during the later stages of selection allowed for escape formation (Wright et al., 2001).

3.2.3. Sequencing and identification of PMI

In order to verify the identity of the amplified fragment, the 1.1 kb PCR product from transgenic samples was cloned and sequenced. The sequence obtained was subjected to BLAST analysis against deposited sequences in the GenBank database. The BLAST results indicate that the DNA sequence of the amplified fragment of oil palm pmı was highly identical (about 98%) to the pmı gene from E. coli (GenBank accession no: M15380).

3.2.4. RT-PCR analysis

In order to study the accumulation of the pmı gene transcripts in oil palm, RT-PCR using reverse transcribed total RNA was performed to confirm pmı expression in the transformed embryos and regenerated transgenic plantlets. Total RNA was isolated from the transgenic oil palm embryos using the method of Zeng and Yang (2002). The integrity of the isolated RNA samples was verified by agarose gel electrophoresis. Results show that 28S rRNA was more abundant than 18S rRNA in all samples, indicating good quality of the extracted samples (Fig. 5). The reverse transcripts were used in PCR amplification using the primer set of PMI-F and PMI-R. In addition, the ribosomal 18S RNA primer was used as the endogenous control for RNA quantification (Fig. 6). Results show that the expected 1.1 kb

![Fig. 7. RT-PCR product from total RNA extracted from oil palm embryos. Two micrograms of purified cDNA were used in all the tissues. The expected fragment (1.1 kb) is indicated by the arrow. Lane M = 1 kb plus DNA ladder marker; W = water (negative control); U = non-transformed (negative control); P = pM3 (positive control); 1–11 = pmı-transformant.](image)

![Fig. 8. Southern hybridization of Sac1-digested DNA isolated from pmı-transgenic oil palm. Genomic DNA was hybridized to 1.1 kb pmı-probe. Lanes M = 1 kb plus DNA ladder, P = pmı gene PCR amplified from PCRIM2-68, 1–20 = DNA isolated from transgenic lines, C = negative control (DNA from non-transformed control plant), U1–U9 = Undigested genomic DNA.](image)
fragment for the pmi gene was obtained for all transgenic plants generated using the pM13, pMl3G, pM11 and pM11G constructs. The bands obtained were not very clear and absent in the non-transgenic plants (Fig. 7). This observation indicates that the pmi transgenes were transcriptionally functional in the regenerated transgenic oil palm plants.

3.2.5. Southern blot analysis

In order to further verify transgene integration in the genome of the transgenic oil palm, isolated genomic DNA from the pmi transplants and non-transgenic control plants was digested with SacI and used in Southern hybridization. SacI has only one site in the vector, located outside the pmi gene. The difference in hybridization patterns among the transgenic lines may indicate different integration locations of the transgene in the oil palm genome. Moreover, the number of bands in each lane may indicate the copy number of the transgene in each event. Fig. 8 showed that positive hybridization signals were found in several transgenic plants derived from the pM13 and pM11 vectors, either undigested or digested DNA. Three copies of the pmi transgene were found in lane 1 and four copies in lane 2. A few lines had most likely single copy insertions of the pmi gene. No signal was detected in the negative control. The Southern hybridization results demonstrate the integration of the pmi gene into the genome of transgenic oil palm.

4. Conclusion

A new selection procedure has been developed for oil palm (E. guineensis Jacq.) transformation. The selection system utilizes the pmi gene from E. coli and mannose as its selection agent substituting sucrose as the carbon source for plant regeneration. The use of mannose as a metabolizable sugar in this positive selection system eliminates the problem of using negative selection systems, such as herbicide or antibiotic resistance genes. The use of different promoters driving the pmi gene does not show much difference in the transformation efficiency. Molecular analyses are then performed to confirm the transgenic status of the transgenic embryoids and plantlets. The results suggest that the mannose-based selection system can be used in oil palm transformation.

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References