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Construction of phosphomannose isomerase (PMI) transformation vectors and evaluation of the effectiveness of vectors in tobacco (*Nicotiana tabacum L*)

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Abstract:
Phosphomannose isomerase (*pmi*) gene isolated from *Escherichia coli* allows transgenic plants carrying it to convert mannose-6-phosphate (from mannose), a carbon source that could not be naturally utilized by plants into fructose-6-phosphate which can be utilized by plants as a carbon source. This conversion ability provides energy source to allow the transformed cells to survive on the medium containing mannose. In this study, four transformation vectors carrying the *pmi* gene alone or in combination with the β-glucuronidase *(gusA)* gene were constructed and driven by either the maize ubiquitin *(Ubi1)* or the cauliflower mosaic virus (CaMV35S) promoter. Restriction digestion, PCR amplification and sequencing were carried out to ensure sequence integrity and orientation. Tobacco was used as a model system to study the effectiveness of the constructs and selection system. PMI11G and pMI3G, which carry *gusA* gene, were used to study the gene transient expression in tobacco. PMI3 construct, which only carries the *pmi* gene driven by CaMV35S promoter, was stably transformed into tobacco using biolistics after selection on 30 g 1^{-1} mannose without sucrose. Transgenic plants were verified using PCR analysis.

Keywords: Transformation vectors, phosphomannose isomerase, *gusA*, biolistics, tobacco.

Abbreviations: PMI/*pmi* - Phosphomannose isomerase, *Ubi1* - Maize ubiquitin promoter, CaMV35S - Cauliflower mosaic virus *35S* promoter, *gusA* - β-glucuronidase GUS reporter gene.

Background:
The production of transgenic plants with novel traits has relied largely on the use of effective selectable marker genes and selection agents. These are generally used in the initial stages of transformation for an early selection of transgenic cells from the majority of untransformed cells **[1].** The frequently used plant selectable marker genes include the *nptII* gene conferring resistance to antibiotics like kanamycin, neomycin and G-418 **[2-4],** the *hph* gene conferring antibiotic hygromycin resistance **[5],** and the *bar* gene conferring resistance to the herbicides containing phosphinothricin as an active compound such as Basta or Bialaphos **[6].**

Due to the public concern on the possible risks posed to human, animal health and the environment by the presence of antibiotic resistance genes in genetically modified plants or their

products, alternative selection systems have been proposed including the use of positive selectable marker genes and selection agents. Among the well studied positive selection marker genes reported are xylose isomerase (*xylA*; **[7])** which utilizes xylose, 2-deoxyglucose-6-phosphate (*DOG*^{R}*1*; **[8])** which confer resistance to 2-Deoxyglucose and phosphomannose isomerase (*pmi***; [9])** which could utilizes mannose as a carbon source. Expression of *pmi* gene isolated from *E. coli* enables transgenic *pmi*-expressing plant cells to convert unmetabolized mannose-6-phosphate into an easily metabolizable fructose-6-phosphate, which serves as a carbon source that improves the energy status and positively influences the growth of transformed cells **[10].** The non-transformed plant cells growing on medium containing only mannose or minimal sucrose will eventually starve and result in cease growth.

PMI enzyme is common in nature. it is found in bacteria *(Escherichia coli)*, yeast *(Saccharomyces cerevisiae)* and mammals *(Amophophallus konjac)*, as well as humans *(Homo sapiens)* **[9, 11-17].** Therefore, the PMI-based selection system employing the *E. coli manA* gene **[9]** will be highly versatile in plants. More importantly, the selection gene product is harmless. The safety assessment for PMI has revealed that purified PMI protein has no unfavourable effects in a mouse toxicity test and does not change glycoprotein profiles in PMI transformed plants **[18-19].**

The mannose-based selection system with phosphomannose isomerase (*pmi*) gene as a selectable marker was first reported by Joersbo *et al*. (1998) for the generation of transgenic sugar beet. PMI has since been shown to be a useful marker in a variety of crops. It enables selection by providing the transgenic cells a metabolic advantage over non-transgenic cells **[20].** Many more important crops were later transformed using this selection system such as cassava **[21],** maize **[22],** wheat **[23],** rice **[24],** pearl millet **[25],** papaya **[26],** sugarcane **[27],** tomato, potato **[28]** and sorghum **[29].** Following the successful utilization of mannose as a suitable selection system for other ultimate crops, this research was embarked with the aim to produce transgenic oil palm (*Elaeis guineensis Jacq*) using mannose as the selection agent. However the transformation vectors carrying the *pmi* gene need to be constructed before the system could be used in oil palm. In this paper, we report the construction of four (4) transformation vectors carrying either *pmi* gene alone or in combination with *gusA* gene driven by either maize ubiquitin or CaMV*35S* promoter. Two of the constructs were tested transiently on tobacco leaves and one was used to stably transform tobacco leave using biolistics approach to evaluate the effectiveness of the construct and gene. These constructs will eventually be used to transform oil palm.

Methodology:

Bacterial strains

E.coli strain DH5α was used in all routine cloning experiments. The preparation of *E. coli* competent cells was performed according to method of Hanahan, 1982 **[30].**

Enzymes and other biochemical reagents

Methods for isolation and manipulation of DNA were as described by Sambrook and Russel, 2001 **[31].** Restriction endonucleases were purchased from Research Biolabs and digestions were performed as recommended by the manufacturer. A QIAquick Gel Extraction Kit (QIAGEN) was used to isolate specific restriction and PCR fragments from agarose gels. DNA ligations and subsequent transformations into competent *Escherichia coli* cells were carried out according to standard protocols **[31].**

Polymerase chain reaction (PCR) amplification

PCR amplification was performed using MJ Research Programmable Thermal Controller (PTC-100™). Amplifications were carried out in 25 µl final reaction volume. Ten (10) ng of plasmid DNA were used as template, depending on experiments. Each PCR reaction contains 2.5 µl 10X PCR buffer (Promega), 3.0 µl 25 mM $MgCl_2$ (Promega), 0.5 µl 10 mM dNTP (Promega), 1.0 µl 10 µM PMI-F (5′-GCG CTA GCC ATG GAA AAA CTC ATT AAC TCA G-3′) and NOS-R (5′-GGA CTA GTG CTA GCG ATC TAG TAA CAT AGA TGA-3′) primers, 0.2 µl 5 U µl^{-1} *Taq* polymerase (Promega) and appropriate amount of template DNA. Total volume was made up with sterile distilled water. The following PCR conditions were used: 95°C for 5 min to denature the DNA template and followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C, and 3 min elongation at 72°C. The reaction was completed with a final 5 min elongation at 72°C. The PCR products were electrophoresed in a 1.2 % agarose gel at 110V for 80 min. The gels were stained with ethidium bromide and visualized under UV light. The PCR products were excised from the agarose gel and purified by using QIAquick PCR Purification Kit (QIAGEN) for cloning.

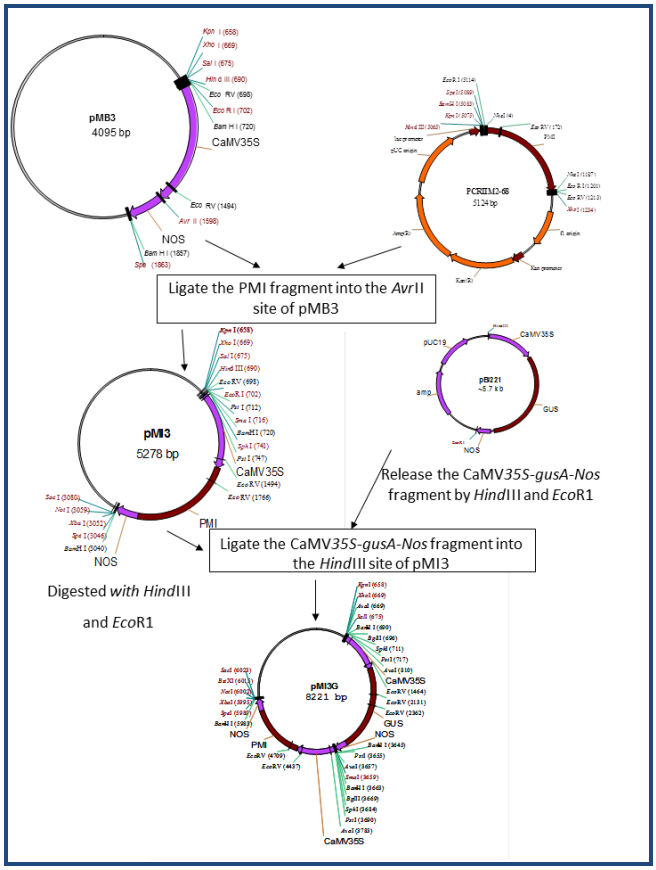

Figure 1: Construction of pMI3 and pMI3G. The PMI fragment was excised from PCRIIM2-68 by *Nhe*I digestion, gel purified

and ligated to the *Avr*II site of pMB3 to create pMI3. The DNA fragment of CaMV*35S-gusA-Nos* was excised from pBI221 by digestion with *Hind*III and *Eco*R1, gel purified and ligated into *Hind*III/ *Eco*R1 site of pMI3 to create pMI3G.

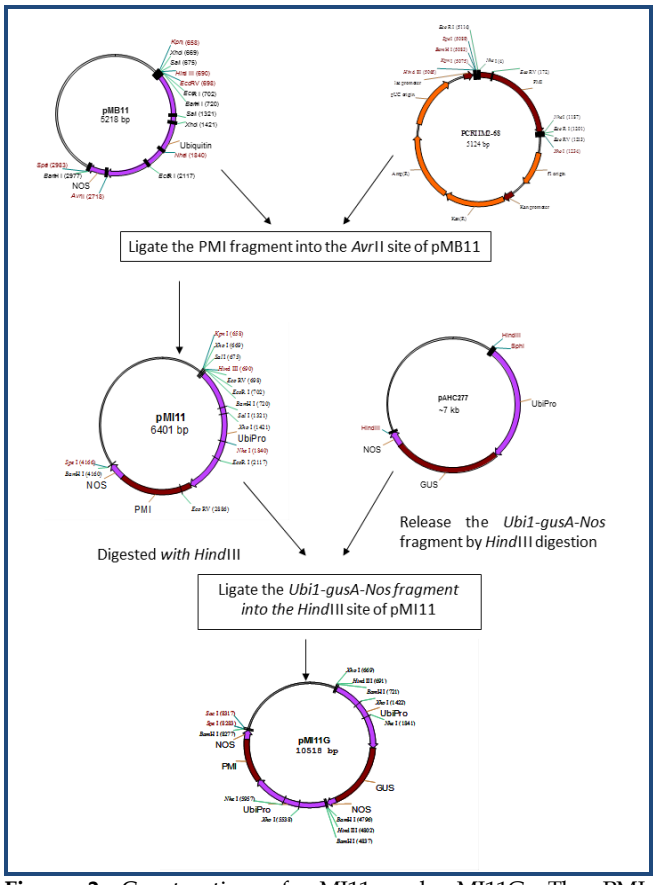

Figure 2: Construction of pMI11 and pMI11G. The PMI fragment was excised from PCRIIM2-68 by *Nhe*I digestion, gel purified and ligated to the *Avr*II site of pMB11 to create pMI11. The DNA fragment of *Ubi1-gusA-Nos* was excised from pAHC27 by *Hind*III digestion, gel purified and ligated into *Hind*III site of pMI11 to create pMI11G.

Vector construction

The pMB3 and pMB11 vectors **[32]** carrying CaMV*35S* and ubiquitin promoters linked to NOS terminator, respectively, were used as backbone vectors for inserting *pmi* gene. The *pmi* gene of *Escherichia coli* strains XL-1 Blue was cloned into PCR2.1TOPO vector by polymerase chain reaction (PCR) using the specific primers (F: 5′-GCG CTA GCC ATG GAA AAA CTC ATT AAC TCA G-3′; R: 5′-GCG CTA GCT TAC AGC TTG TTG TAA ACA CG-3′). The resulted construct was verified by DNA sequencing and designated as PCRIIM2-68. The PCRIIM2-68 plasmid DNA was digested with *NheI* to yield the 1.1 kb fragment. The fragment was gel purified and ligated to the *AvrII* site of pMB3 and pMB11. The ligation mixtures were transformed into DH5α competent cells and plated onto LB agar containing 50 µg ml^{-1} ampicillin, followed by overnight incubation at 37°C. Positive clones were selected by *Bam*HI digestion. The sense orientation of *pmi* gene was confirmed by PCR amplification using primers PMI-F and NOS-R. The two transformation vectors designated as pMI3 and pMI11 were further confirmed by digestion with *Bgl*II, *Eco*R1 and *Hind*III for the presence of DNA fragments with expected size. The plasmid pMI3 contains the *pmi* gene under the control of constitutive promoter, CaMV35S **(Figure 1).** Plasmid pMI11 carries *pmi* gene driven by the ubiquitin promoter **(Figure 2**). The two vectors, pMI3 and pMI11, were further modified by the introduction of *gusA* gene driven by the CaMV35S **(Figure 1)** and ubiquitin promoter **(Figure 2),** respectively. The DNA fragment of CaMV35S-gusA-Nos was excised from pBI221 by digestion with *Hind*III and *Eco*R1, gel purified and ligated into *Hind*III/ *Eco*R1 digested pMI3 to create pMI3G. The ligation mixture was transformed into DH5α competent cells and plated on LB agar containing 50 µg ml^{-1} ampicilin followed by overnight incubation at 37°C. Positive clones were selected by *Hind*III and *Eco*R1 digestion. On the other hand, the *Ubi1-gusA-Nos* fragment was excised from pAHC27 **[33]** by *Hind*III digestion, gel purified and ligated into *Hind*III site of pMI11 to create pMI11G. The ligation mixture was transformed into DH5α competent cells and plated on LB agar containing 50 µg ml^{-1} ampicilin followed by overnight incubation at 37°C. Positive clones were selected by *Hind*III digestion.

Plant material

In this work, tobacco *(Nicotiana tabacum L.)* plant was used as a model system. Leaf discs for transformation work were obtained from four weeks old tobacco plantlets grown *in vitro*. Sterile tobacco leaves were cut into small pieces (2–4 mm) using razor blade and five leaf discs were cultured for each plate. The explants were placed on modified MS medium **[34]** supplemented with 30 g l^{-1} sucrose and 1.0 mg ml^{-1} BAP and cultured in the dark at 25°C. Medium was solidified with 0.8% agar (Sigma, St. Louis, Mo.), and the pH was adjusted to 5.8 before autoclaving (121°C, 20 min). After 30 days, the callus obtained were incubated under 16/8h light/dark conditions and maintained with monthly subculturing for regeneration.

Particle bombardment and selection of transformants

The plasmid DNA was coated onto 1.0 µm gold particles and delivered into leaf discs using parameters previously optimized for Biolistic PDS-1000 Helium device (BioRad) **[35].** Each target plate containing 5 leaf discs was shot twice. After gene delivery, explants were incubated in the dark at 25°C for a month to initiate callus. After one month, the bombarded tissues were transferred onto medium containing 30 g l^{-1} 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 explants were grown under 16/8h light/dark conditions at 25°C and subcultured every four weeks onto fresh selection medium containing 30 g l^{-1} mannose until plantlets were obtained.

Screening transformants:

Transient GUS histochemical assay

Expression of the *gusA* gene was examined using a modified protocol of **[36].** Two *pmi* gene transformation vectors, pMI3G and pMI11G, carrying the *gusA* gene were bombarded into tobacco leaf discs. The experiment was carried out in 5 replicates. The bombarded explants were incubated in the dark at 25°C for 48 h. The explants were later stained overnight (20 h) at 37°C in GUS buffer (0.2M 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^{-1})

dissolved in dimethyl sulfoxide (DMSO). Blue spots were scored optically using a Nikon UFX-DX system.

Polymerase chain reaction (PCR)

For the detection of the *pmi* gene in the regenerated plants, total genomic DNA was isolated from control and transgenic tobacco leaves using the modified method **[37].** PCR amplification was performed using 600 ng of DNA in 25 µl reaction mixtures containing 2.5 µl 10X PCR buffer (Promega), 3.0 µl 25 mM $MgCl_2$ (Promega), 0.5 µl 10 mM dNTP (Promega), 1.0 µl 10 µM appropriate primers; forward and reverse PMI primers (F: 5′-GCG CTA GCC ATG GAA AAA CTC ATT AAC TCA G-3′; R: 5′-GCG CTA GCT TAC AGC TTG TTG TAA ACA CG-3′) and 0.2 µl 5U μl^{-1} Taq polymerase (Promega). PCR was carried out using the PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). A 1.1 kb fragment was expected to be amplified using these PMI primers. The samples were denatured initially at 94°C for 5 min, followed by 34 cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 50°C, and 1 min 5 sec of synthesis at 72°C, with a final extension step of 72°C for 5 min. The PCR products were electrophoresed on a 1.2 % agarose gel at 110V for 80 min. The gels were stained with ethidium bromide and visualized under UV light.

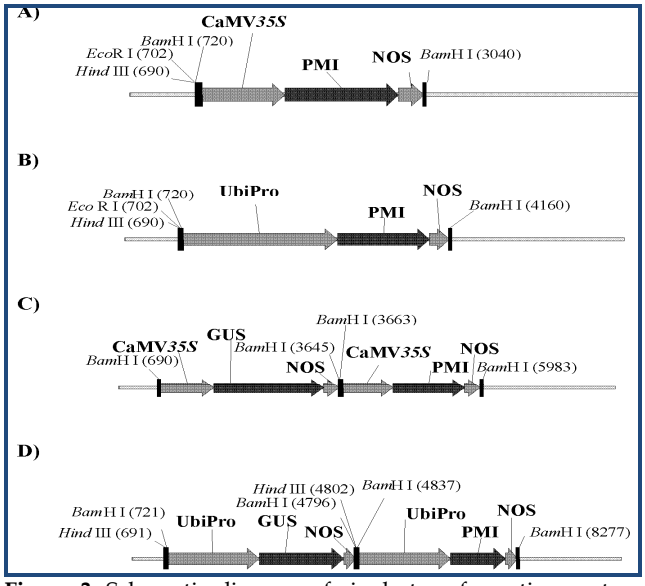

Figure 3: Schematic diagram of single transformation vectors, (A) pMI3; (B) pMI11; (C) pMI3G and (D) pMI11G. The restriction sites and the numbers indicate the approximate position in the vectors.

Discussion:

Construction of PMI transformation vectors

Four vectors carrying the *pmi* gene were engineered to facilitate transformation of oil palm using positive selection via biolistic method. The four vectors constructed for expressing *pmi* gene were designated as pMI3, pMI3G, pMI11 and pMI11G **(Figure 3).** In this study, two constitutive promoters have been chosen to drive the *pmi* gene; CaMV*35S* promoter in plasmid pMI3 and pMI3G and ubiquitin promoter in pMI11 and pMI11G. The most widely employed promoter to drive selectable marker gene is the CaMV*35S* promoter which is strongly expressed in a variety of plant species and tissues **[38].** CaMV*35S* promoter has also been used to drive *pmi* gene in a number of different plant species. The promoter has been shown to be constitutive and highly active especially in dicot plants, such as sugar beet **[10]** and cucumber **[39].** In contrast, ubiquitin promoter has been shown as a suitable promoter for monocot plants **[33, 40-41].** Similar results were obtained for oil palm transformation studies which indicated that ubiquitin promoter is an efficient and stable promoter in oil palm transformed tissues **[42-43].** Previously, ubiquitin promoter has also been shown to drive high levels of *pmi* gene expression in other monocots such as rice **[24, 44],** sugarcane **[27, 45]**, maize **[22],** wheat **[22]** and sorghum **[29].** In all cases, significantly higher transformation frequencies were observed and very few escapes were found. It is believed that the arrest in cell growth of untransformed cells by starvation rather than the necrosis induced by toxic selective agents may contribute to the survival and growth of the transformed cells and high transformation frequencies.

In addition, pMI3G and pMI11G vectors also contained *gusA* gene which is the most widely used reporter gene in plant transformation work. The enzyme utilizes the external substrates 4-methyl umbelliferyl glucuronide (MUG) for measurements of specific activity and 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histological localization **[36].** It is therefore a conditional non-selectable marker gene. GUS expression was used as a reporter to monitor the transformation efficiency. *gusA* gene has been co-transformed with *pmi* selectable marker gene in rice **[24, 44],** rapeseed **[46-47] and** chinese cabbage **[48]** to facilitate the selection of transformed tissues.

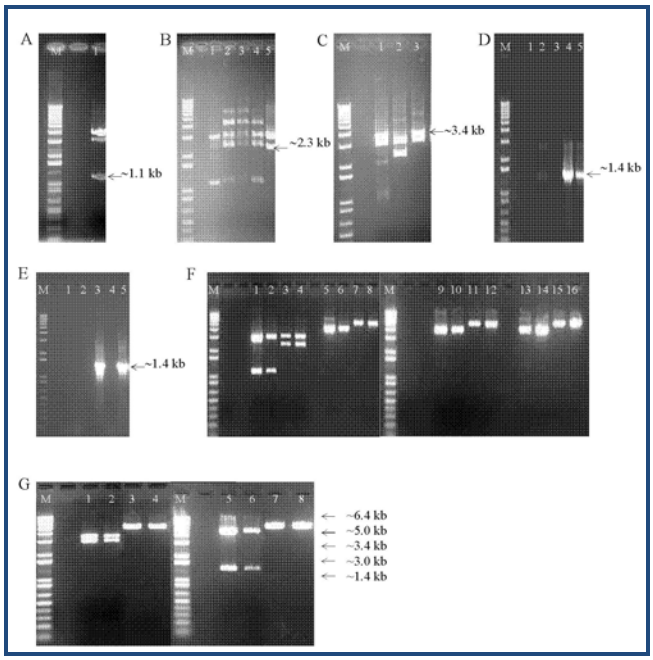

Figure 4: Restriction enzymes and PCR analyses for the construction of pMI3 and pMI11. **(A)** Positive clones for PCRIIM2-68; **(B)** Positive clones for pMI3 vector (lane 5); **(C)** Positive clones for pMI11 vector (lanes 1 and 3); **(D)** Sense orientation of *pmi* gene in pMI3 (lanes 4 and 5); **(E)** Sense orientation of *pmi* gene in pMI11 (lanes 3 and 5); **(F)** Restriction endonuclease analysis of pMB3 (lanes 1, 2, 5, 6, 9, 10, 13 and 14) and pMI3 (lanes 3, 4, 7, 8, 11, 12, 15 and 16) with different

restriction endonucleases (lane 1-4: BamHI; lane 5-8: BglII; lane 9-12: EcoRI; lane 13-16: Hind III); **(G)** Restriction endonuclease analysis of pMI11 with different restriction endonucleases (lane 1-2: BamHI; lane 3-4: BglII; lane 5-6: EcoRI; lane 7-8: HindIII). The size of the fragments in each gel was as predicted. Lane M: 1 kb plus DNA ladder

Initially, for the construction of pMI3 and pMI11 vectors, as shown in **Figure 3A and Figure 3B**, the *pmi* gene was isolated from *Escherichia coli* strains XL-1 Blue by polymerase chain reaction (PCR) to generate a 1.1 kb fragment. The fragment was cloned into PCR2.1TOPO (3.9 kb) to generate PCRIIM2-68 (5.1 kb). PCRIIM2-68 was sequenced for verification. Plasmid was confirmed by digestion using *Nhe*I to yield the 1.1 kb fragment **(Figure 4A).** The 1.1 kb fragment of *pmi* gene was later inserted into the *Avr*II site of pMB3 (4.0 kb) and pMB11 (5.2 kb), respectively. Expected clones were screened by *Bam*HI digestion to cleave the 2.3 kb of CaMV*35S-pmi-Nos* and 3.4 kb of *Ubi1-pmi-Nos* fragment as shown in **Figure 4B and Figure 4C**, respectively. The inserted gene was confirmed to be in sense orientation to the CaMV*35S* and *Ubi1* promoter by PCR analysis using forward (PMI-F) and reverse (NOS-R) primers. A PCR product of approximately 1.4 kb for *pmi-Nos* was amplified as shown in **Figure 4D and Figure 4E.** All plasmids generated (pMI3 and pMI11) were also confirmed by digestion with *Bam*HI, *Bg*lII, *Eco*R1 and *Hind*III to yield fragments with expected size (1.1 kb, 3.3 kb, 4.0 kb, 4.0 kb and 4.0 kb for pMI3; 3.4 kb, 3.0 kb, 6.4 kb, 5.0 kb, 1.4 kb and 6.4 kb for pMI11) as shown in **Figure 4F and Figure 4G.**

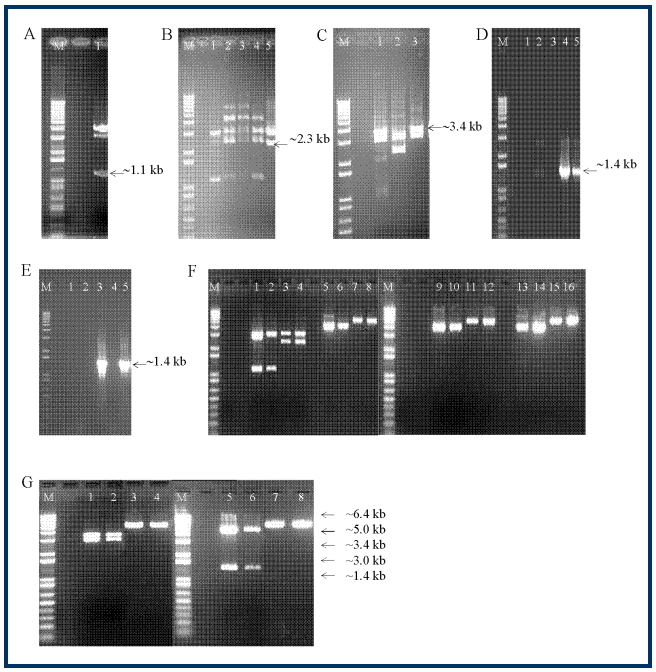

Figure 5: Restriction enzyme analyses for the construction of pMI3G and pMI11G. (A) Purification of DNA fragment (CaMV*35S-gusA-Nos*) from plasmid pBI221; (B) Digestion of pMI3 with *Hind*III and *Eco*R1 (lanes 1 and 2) and pMI11 with *Hind*III (lanes 3 and 4) as cloning vectors; (C) Restriction enzyme analysis of pMI3G with *Hind*III and *Eco*RI digestion; (D) Selection of DNA fragment (*Ubi1-gusA-Nos*) from plasmid pAHC27 with *Hind*III digestion; (E) Restriction enzyme analysis of pMI11G (lane 2) with *Hind*III digestion. The size of the fragments in each gel was as predicted. Lane M: 1 kb plus DNA ladder.

The pMI3G vector as shown in Figure 3C has been constructed by introducing the CaMV*35S-gusA-Nos* fragment from pBI221 **(Figure 5A)** into the *Hind*III and *Eco*R1 site of pMI3 **(Figure 5B).** The reporter gene could be used to monitor transgenic events and visually separate transgenic material from non-transformed material. Selected clones were screened by *Hind*III and *Eco*R1 digestion to cleave the 3.0 kb of CaMV*35S-pmi-Nos* fragment as shown in **Figure 5C.** Similar approach was used to generate pMI11G (10.5 kb) vector shown in **Figure 3D**. *Ubi1-gusA-Nos* fragment from pAHC27 **(Figure 5D**) was introduced into the *Hind*III site in pMI11 **(Figure 5B).** The size of the insert was confirmed by digestion with *Hind*III as shown in **Figure 5E.** All vectors were sequenced to confirm the identity of insert.

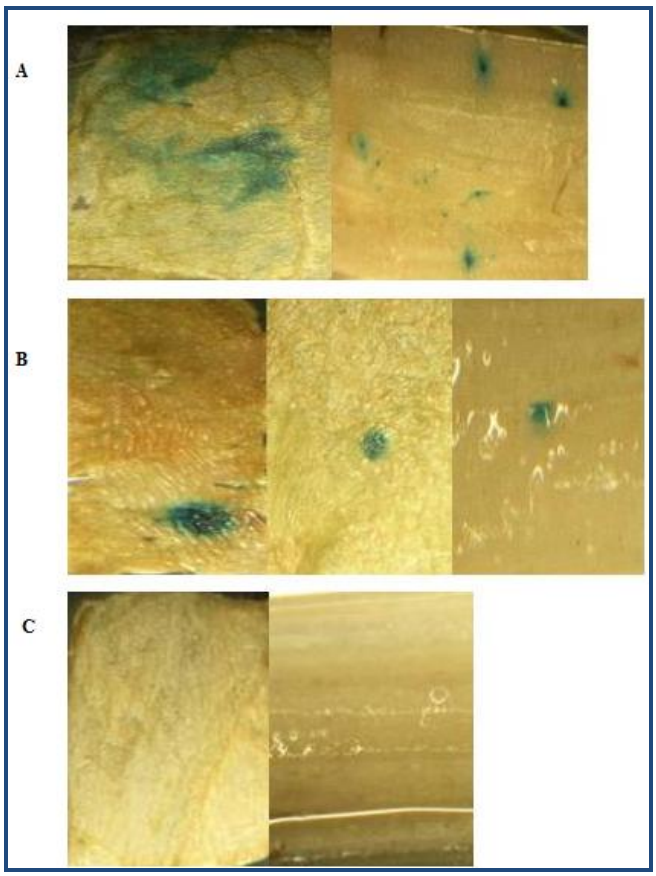

Figure 6: Comparison of transient histochemical *gusA* gene expression in tobacco leaf: **(A)** pMI11G **(B)** pMI3G and **(C)** without DNA.

Transient GUS Histochemical Assay

For transient expression assays, two vectors namely, pMI3G and pMI11G carrying the *pmi* and *gusA* reporter genes, were bombarded into tobacco leaf discs using biolistic PDS/He 1000 device. The *pmi* gene in the 2 vectors was driven by different promoters, namely CaMV*35S* and Ubiquitin, in pMI3G and pMI11G, respectively. Explants were also bombarded with gold particles without DNA as control. Bombarded explants were histochemically assayed for *gusA* expression by staining with X-

Gluc. This was carried out to ensure that the construct carries an effective and functional *gusA* gene. **Figure 6** showed that blue spots were detected as a result of *gusA* activity. No *gusA* activity was observed in control tissues. The result clearly suggested that the blue spots observed were due to introduced gene which confirmed the transgene activity. This indicated that the promoters used were able to drive the *gusA* expression in all tissues. However, the level of *gusA* expression could be further verified by quantification of β-glucuronidase activity.

Selection and regeneration of putative transgenic plants

In this study, callus was induced from leaf discs explants to regenerate plantlets. After bombardment, explants were cultured on selection medium supplemented with 30 g l^{-1} mannose as the carbon source. The calli appeared at the cut ends of expanded tobacco leaf discs after a month of bombardment. Then, primary shoots appeared within a month and subsequently developed into rooted plantlets within 2 to 3 months. Subculture was performed every 3 to 4 weeks on the selection medium. After 3 months, surviving and elongated shoots were developed into rooted plantlets. At 30 g l^{-1} concentration of mannose, shoot and root formation was completely inhibited in untransformed tobacco explants, whereas transgenic tobacco developed normal growth. Cells transformed with the *pmi* gene were able to utilize mannose as a carbon source and grew well. **Figure 7** shows the regeneration of transgenic tobacco tissues from callus formation until regeneration of plantlets complete. The transgenic tobacco plantlets were maintained and regenerated on 30 g l^{-1} mannose prior to confirmation of transgene integration in the genome.

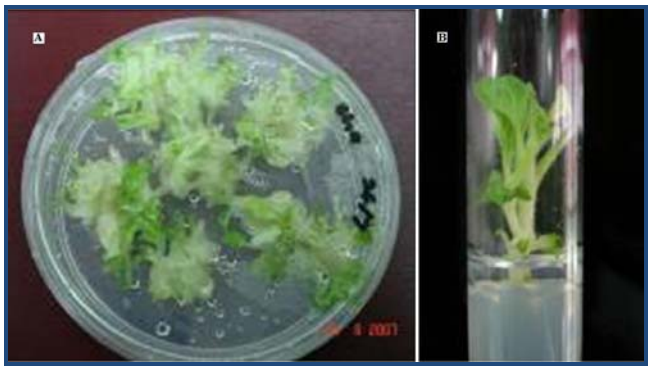

Figure 7: Regeneration of transgenic tobacco plants. **(A)** Tobacco callus and shoot appeared at early stage of selection on media containing mannose and **(B)** shoot development from mannose surviving callus.

PCR Analysis

The integration of *pmi* gene in the regenerated plants was screened using polymerase chain reaction (PCR). The amplification of 1.1 kb fragment will demonstrate the insertion of the *pmi* gene in the genomic DNA. In the PCR analysis of the plantlets transformed with the *pmi* gene, nine out of twelve plantlets amplified the expected 1.1 kb *pmi* gene product **(Figure 8).** The result showed that approximately 75% of tobacco plantlets screened were positive for *pmi* gene. PCR product was also observed for the positive controls (DNA of transformation vector, Lane P), while no signal was detected for the untransformed plantlet (Lane U).

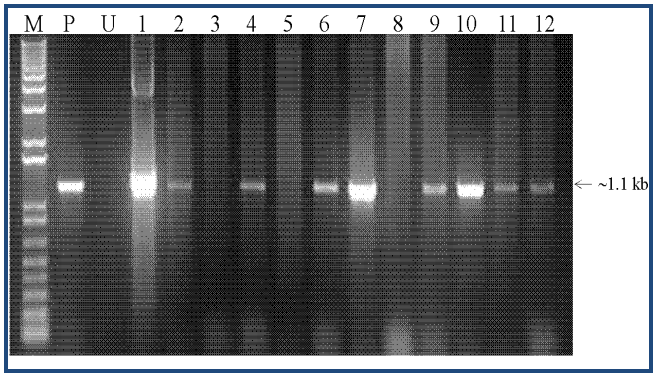

Figure 8: PCR analysis of tobacco plantlets using PMIF-PMIR primers to amplify the *pmi* gene. The expected size (1.1 kb) is indicated by an arrow. Lane M = 1kb plus DNA ladder marker; P= pMI3 (positive control); U = Untransformed (negative control); 1-3 = TB1; 4-6 = TB2; 7-9 = TB3; 10-12 = TB4.

Conclusion:

Four transformation vectors were successfully constructed carrying either *pmi* gene alone or in combination with *gusA* gene driven by either ubiquitin or CaMV35S promoters. The effectiveness of the constructs was evaluated by transient *gusA* gene expression and production of transgenic tobacco plantlets after the use of mannose as a carbon source. The *pmi* gene driven by ubiquitin promoter is being evaluated in oil palm; however, due to the long regeneration of oil palm, the results could not be presented here.

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