

THE USE OF MANNOSE SELECTION SYSTEM FOR GENE TRANSFER IN TOBACCO PLANTS (*Nicotiana tabacum* L.), A MODEL PLANT FOR OIL PALM TRANSFORMATION

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ABSTRACT

A mannose selection system was evaluated for its potential application as a selectable marker in tobacco using biolistic transformation. The above system uses *pmi* gene isolated from *Escherichia coli* that helps transgenic plants expressing it to convert mannose-6-phosphate (from mannose) into a metabolisable carbon source, fructose-6-phosphate. The ability to utilise mannose allows the transformed cells to survive on the medium containing mannose as compared to the untransformed cells. This was achieved by transforming the tobacco leaf discs using a construct carrying the *pmi* and β -glucuronidase (*gusA*) genes which were driven by 35S cauliflower mosaic virus (CaMV35S) promoter. The tobacco leaf discs were cultured on medium supplemented with 30 g litre⁻¹ mannose for callus induction, proliferation and regeneration. The presence of the *pmi* gene was proven by PCR analysis and β -glucuronidase (*gusA*) activity confirmed the expression of *gusA* gene. Results showed that this procedure might be efficient in tobacco and other plants. The transformation procedure presented here, PMI/mannose system for selection of transgenic plants, represents an alternative for the production of transgenic plants under conditions that are safe for human health and the environment.

Keywords: phosphomannose isomerase (PMI), mannose, biolistics, tobacco.

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INTRODUCTION

The process of plant improvement involves the delivery of a foreign gene of interest and a selectable marker gene to effectively and exclusively select transformed cells, calluses and embryos. The most frequently used selectable markers are genes that

include the *nptII* gene conferring resistance to antibiotics like kanamycin, neomycin and G-418; the *hph* gene conferring antibiotic hygromycin resistance; and the *bar* gene conferring resistance to the herbicides containing phosphinothricin as an active compound such as Basta or Bialaphos (Tuteja *et al.*, 2012). However, these common selectable marker genes in transgenic plants can generate environmental and consumer concerns regarding the possible impact of these markers (Zechendorf, 1994). Therefore, alternative selection systems have been developed.

PMI has been shown to be a useful marker in a variety of crops as it enables selection of transgenic cells that have a metabolic advantage over non-

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transgenic cells (Penna *et al.*, 2002). Expression of *pmi* gene from *Escherichia coli* enables transgenic *pmi*-expressing plant cells to convert the plant un-metabolised mannose-6-phosphate (from mannose) into an easily metabolisable fructose-6-phosphate, which serves as a carbon source that improves the energy status of transgenic tissue (Joersbo *et al.*, 1998). Moreover, the evaluation study on PMI allergenicity potential indicated the lack of attributes associated with known oral allergens (Privalle, 2002). The PMI/mannose selection system has been adapted in many other plant species, such as *Arabidopsis* (Todd and Tague, 2001), rice (Lucca *et al.*, 2001; He *et al.*, 2004), maize (Wang *et al.*, 2000), wheat (Wright *et al.*, 2001; Gadaleta *et al.*, 2006), orange (Boscariol *et al.*, 2003), tomato (Sigareva *et al.*, 2004), potato (Briza *et al.*, 2008), grape (Vaccari and Martinelli, 2009), sorghum (Gao *et al.*, 2005), papaya (Zhu *et al.*, 2005), cucumber (He *et al.*, 2006), apple (Degenhardt *et al.*, 2006), sugar-cane (Jain *et al.*, 2007), cabbage (Min *et al.*, 2007) and lettuce (Briza *et al.*, 2010).

In this study, we attempted to evaluate and use this mannose-based selection system in tobacco plants and to further extend its application into oil palm. All previous studies on oil palm transformation have used an antibiotic hygromycin (hygromycin phosphotransferase [*hpt*]) and the herbicide Basta (phosphinothricin acetyltransferase [*bar*]) (Parveez and Christou, 1998; Abdullah *et al.*, 2005) as selectable markers. Since these selectable markers have generated widespread public concern, mannose-based selection system is an alternative method for obtaining transgenic oil palm. Mannose selection offers a more environmentally system as sugar metabolism, which is considered ecologically neutral, will also improve the transformation efficiency.

This article focused on biolistic transformation of tobacco plant using a construct containing *pmi* gene and followed by confirmation of the transgenic tobacco plants status using molecular analysis.

MATERIALS AND METHODS

Plant Material

Leaf discs from four weeks old *in vitro* tobacco plantlets *Nicotiana tabacum* from the cultivar L. NT1 provided by the Malaysian Rubber Board were used as explants. These plantlets were generated from sterilised-mature tobacco leaves which were cut into small pieces (2-4 cm) using razor blade. The leaf discs were then placed on MS medium (Murashige and Skoog, 1962) supplemented with 30 g litre⁻¹ sucrose and 4.44 µM BAP, and cultured in the dark at 25°C. After 30 days, the calli obtained were incubated under 16/8 hr light conditions and cultivated until plantlet regenerated.

Determination of Mannose Concentration for Selection of Transformants

Tobacco leaf discs were transferred onto MS medium supplemented with various combinations of mannose and sucrose [mannose (g litre⁻¹): sucrose (g litre⁻¹); 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, 30:0] by maintaining total sugars at 30 g litre⁻¹. Five plates were used for each treatment. In each plate, five leaf discs were placed onto the solidified media and incubated in the dark at 25°C for a month, and then the discs were transferred onto fresh media plates containing the same concentrations of mannose and sucrose and half of them were placed under 16/8 hr light for callusing or shoot regeneration with the different treatments. After two months, the shoot formation rate was determined by measuring the height of plantlets that are present or survived in the shoot induction medium. The combinations that were more restrictive for shoot regeneration were chosen to be tested in transformation experiments with *pmi*/mannose selection.

Plasmid, Particle Bombardment and Selection of Transformants

Plasmid pMI3G (~8.0 kb) (Bahariah *et al.*, 2012), carrying *pmi* gene which was isolated from *E. coli* strains XL-1 Blue, *gusA* gene under the control of the 35S cauliflower mosaic virus (CaMV35S) promoter, and the nopaline synthase (NOS) terminator (Figure 1), was coated onto 1.0 µm gold particles and delivered into leaf discs using parameters previously optimised for Biolistic PDS-1000 Helium device (BioRad) (Parveez *et al.*, 1997; 1998). The functionality of pMI3G vector was assessed by GUS staining using a modified protocol of Jefferson (1989). The bombarded explants were incubated in the dark at 25°C for 48 hr. The explants were later stained overnight (20 hr) 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 sulphoxide (DMSO). Blue spots were scored optically using a Nikon UFX-DX system. Meanwhile, for the regeneration of transgenic tobacco plants, the bombarded tissues were transferred onto medium containing 30 g litre⁻¹ mannose and incubated in the dark at 25°C for a month to initiate callus, and then in light condition for proliferation until plantlets were obtained.

Polymerase Chain Reaction (PCR) Analysis

For the detection of the *pmi* gene in the regenerated transgenic plants, total genomic DNA was isolated from untransformed and transgenic tobacco leaves according to the method of Doyle and Doyle

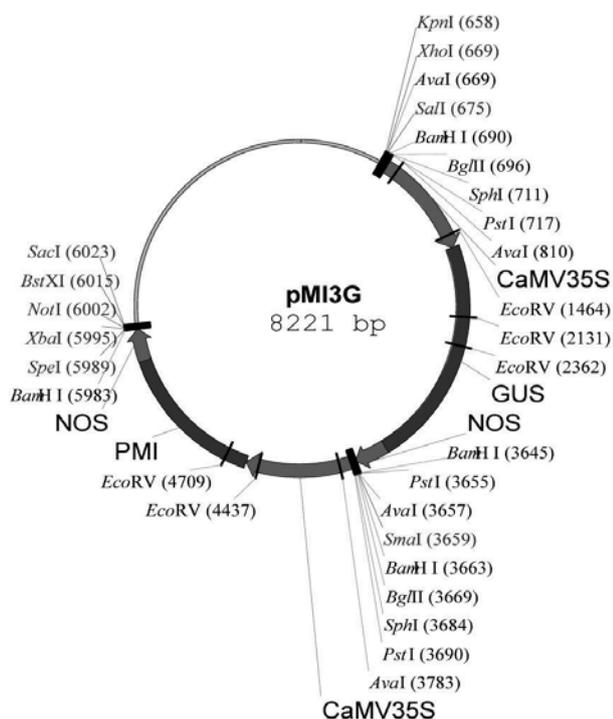


Figure 1. Schematic map of the pMI3G construct. Arrow indicates the orientation of each DNA fragment assembled, CaMV35S: cauliflower mosaic virus 35S promoter, GUS: gene for β -glucuronidase, NOS: terminator of nos (nopaline synthase) gene, PMI: gene for phosphomannose isomerase.

(1987). PCR amplification was performed using 600 ng of genomic DNA in 25 μ l reaction mixture containing 2.5 μ l 10X PCR buffer (Promega), 3.0 μ l 25 mM MgCl₂ (Promega), 0.5 μ l 10 mM dNTP (Promega), 1.0 μ l 10 μ M appropriate primers; forward and reverse PMI primers (PMI-F: 5'-GCGCTAGCCATGGAAAAC TCATTA ACTCAG-3'; PMI-R: 5'-GCGCTAGCTTAC AGCTTGTGTAACACG-3') and 0.2 μ l 5U/ μ l Taq polymerase (Promega). PCR was carried out using the PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). A 1.1 kb band was expected to be amplified. The samples were denatured initially at 94°C for 5 min, followed by 34 cycles of 1 min denaturation at 94°C, 1 min of primer annealing at 50°C, and 1 min 5 s 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 % (w/v) agarose gel at 110V for 80 min. The gels were stained with ethidium bromide (1 μ g ml⁻¹) and visualised under UV light.

Sequencing and Identification of PMI Transgene

The PCR products were confirmed by direct sequencing using an automated DNA sequencer (ABI PRISM Model 377 Version 3.4) with the PMI-R and PMI-F primers. Analysis of DNA sequences was carried out using VectorNTI software (Invitrogen). The analysis included the removal of unreadable and vector sequences, and DNA sequence alignment.

A protein homology search against the GenBank databases was performed using BLAST 2.0 (Altschul *et al.*, 1997).

RESULTS AND DISCUSSION

Optimal Mannose Concentration for Selection of Transformants

In selection using mannose, the untransformed cells could convert mannose to mannose-6-phosphate but are unable to isomerise mannose-6-phosphate to fructose-6-phosphate and are thus deprived of a carbon source and become depleted of phosphorous (Barb *et al.*, 2003). This leads to the retardation of tissue growth for PMI-limited plant species. If other carbohydrate sources such as sucrose and glucose are not present in the medium, no further growth of those plants will be observed. Therefore, optimal mannose concentration for selection of transgenic PMI-limited plants species were determined by a combination of sucrose and glucose. The addition of sucrose to the selection medium may modulate the inhibitory effect of mannose on organogenesis; it has allowed better recovery of transgenic shoots in different plant species. For examples, the concentrations of 1.25 g litre⁻¹ mannose plus 30 g litre⁻¹ sucrose for sugar beet (Joersbo *et al.*, 1999), 5 g litre⁻¹ mannose plus 20 g litre⁻¹ sucrose for wheat (Wright *et al.*, 2001), 10 g litre⁻¹ mannose plus 5 g litre⁻¹ glucose for tomato (Sigareva *et al.*, 2004), 30 g litre⁻¹ mannose for papaya (Zhu *et al.*, 2005), 1-10 g litre⁻¹ mannose plus 5-30 g litre⁻¹ sucrose for apple (Degenhardt *et al.*, 2006) and 12 g litre⁻¹ mannose plus 5 g litre⁻¹ sucrose for citrus (Ballester *et al.*, 2008) have been used for selection of transformants. This indicates that the transformation efficiency using the PMI/mannose system may vary depending on the species or cultivar.

In this study, an increase in mannose concentration resulted in a lower number of explants with calli (Figure 2). At 30 g litre⁻¹ mannose, only two out of five tobacco leaf discs initiated minimal amount of calli (Figure 2a) compared to 0 to 25 g litre⁻¹ mannose (Figures 2b to 2g). Those calli were further cultivated for two months and retardation in shoot length was observed in the media containing increasing concentrations of mannose. At 30 g litre⁻¹ mannose, there was an average of 70% retardation in shoot length (Table 1). The length of shoot ranged from 15.5 cm at 0:30 g litre⁻¹ mannose:sucrose to 4 cm at 30:0 g litre⁻¹ mannose:sucrose. Since the mannose selection system involves the plant metabolism, the effect of light for plant growth was investigated. Plantlets were initiated from tobacco explants both in darkness and in light and resulted in similar growth rate as shown in Figure 3, but with different morphology and physiology. Plants grown under

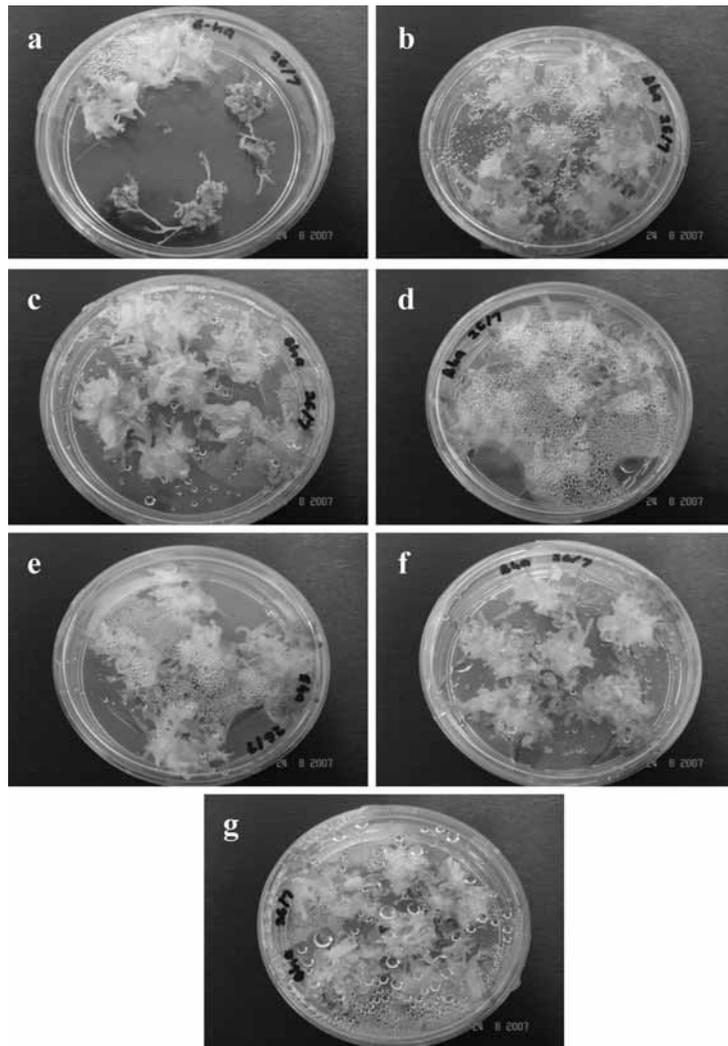


Figure 2. Determination of mannose concentration for tobacco explants to produce calli. Different amounts of calli were obtained at different concentrations of mannose: sucrose; (a) 30:0; (b) 0:30; (c) 5:25; (d) 10:20; (e) 15:15; (f) 20:10 and (g) 25:5 g litre⁻¹.

TABLE 1. EFFECT OF VARYING MANNOSE CONCENTRATION ON SHOOT FORMATION. THE MEANS FROM THREE SHOOTS/TREATMENT WERE COMPUTED

Medium	Mannose (g litre ⁻¹)	Sucrose (g litre ⁻¹)	Length of shoot (cm) Mean score ± std error	
			Light	Dark
A	0	30	12.0 ± 0.03	15.5 ± 0.03
B	5	25	10.5 ± 0.03	16.0 ± 0.06
C	10	20	10.6 ± 0.03	14.0 ± 0.00
D	15	15	9.6 ± 0.05	7.4 ± 0.06
E	20	0	9.5 ± 0.06	7.5 ± 0.03
F	25	5	7.5 ± 0.03	5.5 ± 0.05
G	30	0	5.0 ± 0.000	4.0 ± 0.000

Note: Values represent the means ±S.E of three replications after five months.

light with full mannose concentration (30 g litre⁻¹ mannose: 0 g litre⁻¹ sucrose) only achieved one-third of height of the control plant. Plants grown in dark also showed similar response towards different concentrations of mannose. As expected, plants grown under light are more greenish and bushy but plants grown under dark condition showed symptoms of chlorosis. Nevertheless, all plants seem to survive in all treatments suggesting that no significant difference in plant growth was observed when cultured either in the light or dark conditions. The results showed that light play an important role in plant photosynthesis by regulating the plant growth, metabolism and cells differentiation. In dark condition where photosynthesis was absent, carbohydrate source was assumed to be minimal.

The decrease in shoot growth in the presence of higher concentration of mannose in this study indicates that mannose can be used as a selection agent for tobacco with the dosage at 30 g litre⁻¹ of mannose. Tobacco, as soyabean and other leguminous plants, belongs to non-PMI-limited species, thus a high amount of mannose is required for selection of transformants. Song *et al.* (2010) reported that wild type tobacco plants (*Nicotiana tabacum* cv. Samsun) were able to utilise mannose as a carbon source for plant regeneration. A high mannose level of 50 g litre⁻¹ did not inhibit shoot regeneration from leaf explants and even for seed germination. *Nicotiana tabacum* cv. Samsun showed high mannose tolerance probably due to its endogenous PMI.

Plant Transformation and Regeneration of Transgenic Tobacco Plants

The explants were maintained on selection medium supplemented with 30 g litre⁻¹ mannose as the carbon source. Plantlet regeneration using leaf disc explants was used for callus induction (Figure 4a). The calli appeared at the cut ends of expanded tobacco leaf discs after a month of bombardment (Figure 4b). Then primary shoots appeared within a month (Figure 4c). Subculture was performed every three to four weeks on the selection medium. After three months, surviving and elongated shoots (Figure 4d) developed into rooted plantlets (Figures 4e and 4f). This result showed that at 30 g litre⁻¹ concentration of mannose, shoot and root formation was completely inhibited in untransformed tobacco explants, whereas transformed tobacco developed normal growth. Cells transformed with the *pmi* gene were able to utilise mannose as a carbon source and grew well. The transgenic cells enjoyed a metabolic advantage compared to the non-transgenic cells that were unable to metabolise mannose-6-phosphate.

Explants were also bombarded with gold particles without DNA as a control. Bombarded explants were histochemically assayed for *gusA*

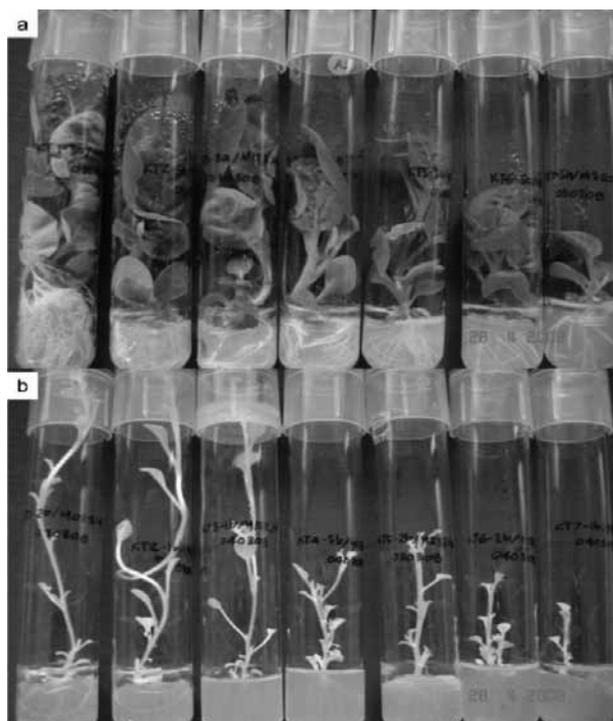


Figure 3. Effect of light and dark on plantlets regeneration. Comparison of tobacco plantlets production on media containing various concentrations of mannose: sucrose; from left, 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, 30:0 g litre⁻¹. Plants incubated in the light (a) and in the dark (b) after five months at 25°C. Shoot height decreased with increasing mannose content in the medium.

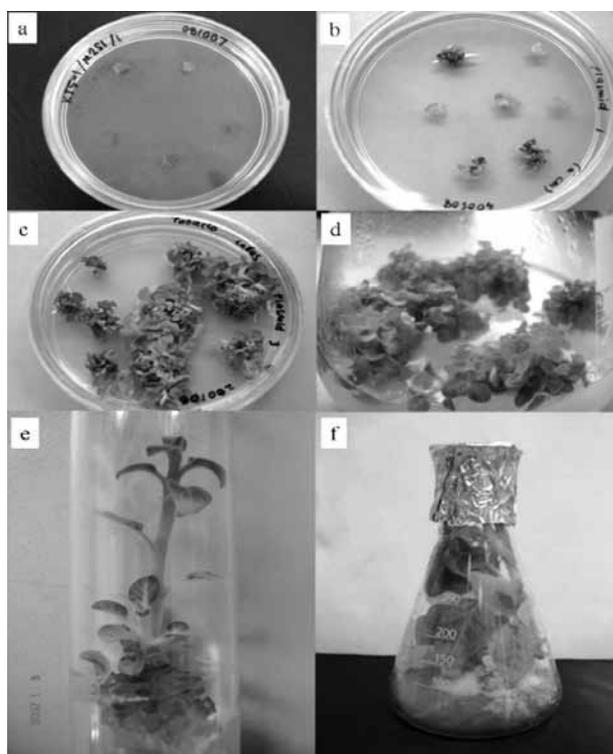


Figure 4. Regenerated transgenic tobacco plants expressing *pmi* gene. Development and regeneration of transgenic tobacco plants by culturing on medium containing 30 g litre⁻¹ mannose, (a) tobacco leaf discs; (b) callus appeared at cut ends of leaf discs; (c) shoot appeared at early stage; (d) primary shoots; (e) elongated shoot and (f) tobacco plantlet at rooting stage.

expression by staining with X-Gluc. This was carried out to simply ensure that the biolistic could be used to transform the tobacco explant. Figure 5 shows that blue spots were detected in tissues bombarded with pMI3G. No *gusA* activity was observed in control tissues. Histochemical staining revealed that blue spots observed indicate the *gusA* gene expression. The result also indicated that the promoter used was able to drive the *gusA* expression in all tissues.

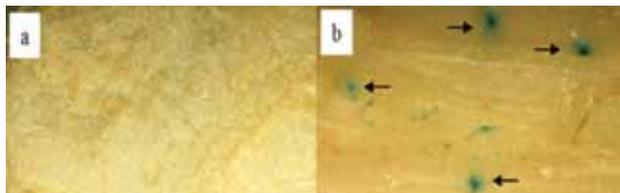


Figure 5. Comparison of transient histochemical *gusA* gene expression in tobacco leaf. (a) Tobacco leaf disc bombarded without DNA; (b) tobacco leaf disc bombarded with plasmid DNA pMI3G. Arrows indicate the blue spots demonstrating the expression of *gusA*.

Molecular Analysis of Transgenic Tobacco Plants

Isolated genomic DNA from the putative transformed plants and untransformed plants were used as a PCR template. Primers for the *pmi* gene were designed to amplify 1.1 kb fragment of the *pmi* gene (Figure 1). 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 6).

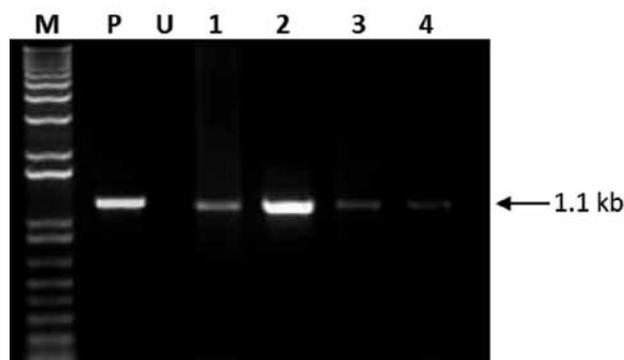


Figure 6. Polymerase chain reaction (PCR) analysis of transgenic tobacco plants using PMIF-PMIR primers to amplify the *pmi* gene. (M) 1kb plus DNA ladder marker (Invitrogen, USA); (U) negative control (untransformed tobacco plants); (P) positive control (plasmid DNA pMI3G); (1-4) transformed plantlets. Band with expected size (1.1 kb) is indicated by an arrow.

Furthermore, direct sequencing approach has also been used to confirm the identity of *pmi* gene amplified by PCR from the transgenic samples. The sequence obtained was subjected to BLAST analysis against deposited sequences in the GenBank database (Figure 7). Based on PCR analysis of *pmi* gene on transgenic tobacco leaf samples, it could be suggested that around 75% of the transformed plants showed integration of the *pmi* gene (Table 2). The BLAST results indicated that the DNA sequence of the amplified fragment from genomic DNA of transgenic tobacco is highly similar to *pmi* gene sequence which was isolated from *E. coli* (Genbank accession No. M15380) at about 98%. As PCR is the most rapid method for isolating a particular sequence from the genome, the fastest way to sequence the same locus from a large number of different species would be to directly sequence PCR amplified products (Ritland *et al.*, 1993).

CONCLUSION

An alternative selection procedure has been evaluated for tobacco (*Nicotiana tabacum* L.) transformation. It is a positive selection system with *pmi* as the selectable marker gene and mannose as the selection agent. Transgenic events can be identified initially by PCR for indicating the presence of the *pmi* gene. Our study addresses the concern on the use of conventional antibiotic resistance genes in the development of transgenic plants, which cause

TABLE 2. PMI GENE INTEGRATION EFFICIENCY IN TRANSGENIC TOBACCO SELECTED ON 30 g litre⁻¹ MANNOSE SELECTION MEDIUM

Sample	No. of tested (A)	No. of PCR positive for <i>pmi</i> gene (B)	PMI gene integration efficiency (B/A, %)
Plantlet	12	9	75

	1	50
E.coli pmi	(1)	ATGCAAAACTCATTAACTCAGTGCAAAACTATGCCTGGGGCAGCAAAAC
Transgenic pmi	(1)	-----CAGCAAAAC
Consensus	(1)	CAGCAAAAC
		100
E.coli pmi	(51)	GGCGTTGACTGGAACITTTATGGTATGGGAAATCCGTCCAGCCAGCCGAT
Transgenic pmi	(10)	GGCGTTGACTGGAACITTTATGGTATGGGAAATCCGTCCAGCCAGCCGAT
Consensus	(51)	GGCGTTGACTGGAACITTTATGGTATGGGAAATCCGTCCAGCCAGCCGAT
		101 150
E.coli pmi	(99)	GGCCGAGCTGTGGATGGGCGCACATCCGAAAGCAGTTCACGAGTGCAGA
Transgenic pmi	(58)	GGCCGAGCTGTGGATGGGCGCACATCCGAAAGCAGTTCACGAGTGCAGA
Consensus	(101)	GGCCGAGCTGTGGATGGGCGCACATCCGAAAGCAGTTCACGAGTGCAGA
		151 200
E.coli pmi	(149)	ATGCCCGGGAGATATCGTTTCACTGCGTATGTGATTGAGAGTGATAAA
Transgenic pmi	(108)	ATGCCCGGGAGATATCGTTTCACTGCGTATGTGATTGAGAGTGATAAA
Consensus	(151)	ATGCCCGGGAGATATCGTTTCACTGCGTATGTGATTGAGAGTGATAAA
		201 250
E.coli pmi	(199)	TCGACTCTGCTCGGAGAGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
Transgenic pmi	(158)	TCGACTCTGCTCGGAGAGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
Consensus	(201)	TCGACTCTGCTCGGAGAGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
		251 300
E.coli pmi	(249)	CCTGTTCAAAGTATTATGGCGCAGCAGCCACTCCATTCAGGTTCAATC
Transgenic pmi	(208)	CCTGTTCAAAGTATTATGGCGCAGCAGCCACTCCATTCAGGTTCAATC
Consensus	(251)	CCTGTTCAAAGTATTATGGCGCAGCAGCCACTCCATTCAGGTTCAATC
		301 350
E.coli pmi	(299)	CAAAACAACCAATTCGAAATCGGTTTTGCCAAAGAAATGCCCGAGGT
Transgenic pmi	(258)	CAAAACAACCAATTCGAAATCGGTTTTGCCAAAGAAATGCCCGAGGT
Consensus	(301)	CAAAACAACCAATTCGAAATCGGTTTTGCCAAAGAAATGCCCGAGGT
		351 400
E.coli pmi	(349)	ATCCCGATGGATGCGCGGAGCGTAACTATAAAGTCCCTAACCAAGCC
Transgenic pmi	(308)	ATCCCGATGGATGCGCGGAGCGTAACTATAAAGTCCCTAACCAAGCC
Consensus	(351)	ATCCCGATGGATGCGCGGAGCGTAACTATAAAGTCCCTAACCAAGCC
		401 450
E.coli pmi	(399)	GGAGCTGGTTTTGCGCTGACGCTTTCTTCCGATGAACGGGTTTCGTG
Transgenic pmi	(358)	GGAGCTGGTTTTGCGCTGACGCTTTCTTCCGATGAACGGGTTTCGTG
Consensus	(401)	GGAGCTGGTTTTGCGCTGACGCTTTCTTCCGATGAACGGGTTTCGTG
		451 500
E.coli pmi	(449)	AATTTCCGAGATTGCTCCCTACTCCAGCCGTCGAGGTGCACATCCG
Transgenic pmi	(408)	AATTTCCGAGATTGCTCCCTACTCCAGCCGTCGAGGTGCACATCCG
Consensus	(451)	AATTTCCGAGATTGCTCCCTACTCCAGCCGTCGAGGTGCACATCCG
		501 550
E.coli pmi	(499)	GCGATTGCTCACITTTTACACAGCCGCTGATGCCGAACGTTAAAGCAACT
Transgenic pmi	(458)	GCGATTGCTCACITTTTACACAGCCGCTGATGCCGAACGTTAAAGCAACT
Consensus	(501)	GCGATTGCTCACITTTTACACAGCCGCTGATGCCGAACGTTAAAGCAACT
		551 600
E.coli pmi	(549)	GTTCGCCAGCCTGTTGAATATGCAGGTTGAAGAAATCCCGCGGCTGG
Transgenic pmi	(508)	GTTCGCCAGCCTGTTGAATATGCAGGTTGAAGAAATCCCGCGGCTGG
Consensus	(551)	GTTCGCCAGCCTGTTGAATATGCAGGTTGAAGAAATCCCGCGGCTGG
		601 650
E.coli pmi	(599)	CGATTTTAAATCGGCCCTCGATAGCAGCAGGTTGAACCGTGCAAAACG
Transgenic pmi	(558)	CGATTTTAAATCGGCCCTCGATAGCAGCAGGTTGAACCGTGCAAAACG
Consensus	(601)	CGATTTTAAATCGGCCCTCGATAGCAGCAGGTTGAACCGTGCAAAACG
		651 700
E.coli pmi	(649)	ATTCTGTTAATTTCTGAAATTTACCCGGAAGCAGCGGTTCTGTTCCGCC
Transgenic pmi	(608)	ATTCTGTTAATTTCTGAAATTTACCCGGAAGCAGCGGTTCTGTTCCGCC
Consensus	(651)	ATTCTGTTAATTTCTGAAATTTACCCGGAAGCAGCGGTTCTGTTCCGCC
		701 750
E.coli pmi	(699)	GCTATTGCTGAATGTGGTGAATTTGAACCCCTGGCGAAGCGATGTTCTGCT
Transgenic pmi	(658)	GCTATTGCTGAATGTGGTGAATTTGAACCCCTGGCGAAGCGATGTTCTGCT
Consensus	(701)	GCTATTGCTGAATGTGGTGAATTTGAACCCCTGGCGAAGCGATGTTCTGCT
		751 800
E.coli pmi	(749)	TCGCTGAACACCGCAGCCTTACCTGCAAGCGGTGGCGCTGGAAGTGATG
Transgenic pmi	(708)	TCGCTGAACACCGCAGCCTTACCTGCAAGCGGTGGCGCTGGAAGTGATG
Consensus	(751)	TCGCTGAACACCGCAGCCTTACCTGCAAGCGGTGGCGCTGGAAGTGATG
		801 850
E.coli pmi	(799)	GCAAACTCCGATAACGCTGCTGCGTGGCGGTTGAGCGCTAAATACATTGA
Transgenic pmi	(758)	GCAAACTCCGATAACGCTGCTGCGTGGCGGTTGAGCGCTAAATACATTGA
Consensus	(751)	TCGCTGAACACCGCAGCCTTACCTGCAAGCGGTGGCGCTGGAAGTGATG
		801 850
E.coli pmi	(799)	GCAAACTCCGATAACGCTGCTGCGTGGCGGTTGAGCGCTAAATACATTGA
Transgenic pmi	(758)	GCAAACTCCGATAACGCTGCTGCGTGGCGGTTGAGCGCTAAATACATTGA
Consensus	(801)	GCAAACTCCGATAACGCTGCTGCGTGGCGGTTGAGCGCTAAATACATTGA
		851 900
E.coli pmi	(849)	TATTCGGAACTGGTGGCAATGTGAAATTCGAAAGCAAAACCGGCTAAC
Transgenic pmi	(808)	TATTCGGAACTGGTGGCAATGTGAAATTCGAAAGCAAAACCGGCTAAC
Consensus	(851)	TATTCGGAACTGGTGGCAATGTGAAATTCGAAAGCAAAACCGGCTAAC
		901 950
E.coli pmi	(899)	AGTTGTTGACCCAGCCGTTGAACAGGTGCGAGACTGGACTCCCGATT
Transgenic pmi	(858)	AGTTGTTGACCCAGCCGTTGAACAGGTGCGAGACTGGACTCCCGATT
Consensus	(901)	AGTTGTTGACCCAGCCGTTGAACAGGTGCGAGACTGGACTCCCGATT
		951 1000
E.coli pmi	(949)	CCAGTGGATGATTTGCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
Transgenic pmi	(908)	CCAGTGGATGATTTGCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
Consensus	(951)	CCAGTGGATGATTTGCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
		1001 1050
E.coli pmi	(999)	CACCATAGCCAGCAGAGTCCGCCAATTTGTTCTGCGTCAAGGGCGATG
Transgenic pmi	(958)	CACCATAGCCAGCAGAGTCCGCCAATTTGTTCTGCGTCAAGGGCGATG
Consensus	(1001)	CACCATAGCCAGCAGAGTCCGCCAATTTGTTCTGCGTCAAGGGCGATG
		1051 1100
E.coli pmi	(1049)	CAACGTTGTGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGTTGAATCA
Transgenic pmi	(1008)	CAACGTTGTGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGTTGAATCA
Consensus	(1051)	CAACGTTGTGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGTTGAATCA
		1101 1150
E.coli pmi	(1099)	CGGTTTATGCCCCCAACGAATCAGCGGTGACTGCAAAAGCCACGGCCG
Transgenic pmi	(1058)	CGGTTTATGCCCCCAACGAATCAGCGGTGACTGCAAAAGCCACGGCCG
Consensus	(1101)	CGGTTTATGCCCCCAACGAATCAGCGGTGACTGCAAAAGCCACGGCCG
		1151 1178
E.coli pmi	(1149)	TTTAGCCGCTTTTACAAACAAGCTGTAA
Transgenic pmi	(1098)	-----
Consensus	(1151)	-----

Figure 7. Comparison of DNA sequence between tobacco pmi and its corresponding pmi gene of E. coli. DNA sequence of the amplified fragment from genomic DNA of transgenic tobacco is highly similar to pmi gene sequence isolated from E. coli at about 98% (Genbank accession No. M15380).

widespread public concern because of inadequate knowledge of the agent's impact on the environment and on human health.

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