CHAPTER 1

GENERAL INTRODUCTION

1.1 Oil palm (*Elaeis guineensis* Jacq)

Oil palm (*Elaeis guineensis* Jacq, Palmae, 2n=32) is the most important commodity crop in Malaysia. It is a tall palm, with height of 8.3-20 m. Mature trees are single-stemmed, and perennial monocotyledonous tree with long generation period of about 7-10 years. The leaves are pinnate, and reach between 3-5 m long. The flowers are produced in dense clusters where each individual flower is small, with three sepals and three petals. The fruit takes 5-6 months to mature from pollination to maturity and it comprises of the fleshy mesocarp producing palm oil, which is used mainly for its edible properties and the kernel produces palm kernel oil, which has wide application in the oleochemical industry. *Elaies guineensis* is native to West Africa and was first introduced to Brazil and other tropical countries in the 15th Century by the Portuguese (Corley et al., 1986). In 1948, the Dutch brought *dura* species seeds from West Africa to Indonesia resulting in four seedlings planted in Buitenzorg Botanical Garden (now Bogor), Indonesia. The progenies from these seedlings were planted as ornamentals in Deli and became known as Deli *Dura*. Then on, the oil palm was sent to the Botanical Gardens, Singapore in 1875, and subsequently brought to Malaya (now West Malaysia) in 1878 as an ornamental plant and for landscaping (Hartley, 1988). The first commercial planting started only in 1917 at Tennamaran Estate, Selangor (Corley and Tinker, 2003).

In Malaysia, the trees planted are mainly the *tenera* variety, a hybrid between the *dura* and *pisifera*. The *tenera* variety yields about 4 to 5 tonnes of crude palm oil (CPO) per

hectare per year and about 1 tonne of palm kernels. The oil palm is most efficient, requiring only 0.25 hectares to produce one tonne of oil while soybean, sunflower and rapeseed need 2.15, 1.50 and 0.75 hectares, respectively. The rapid increase in plantation area in Malaysia from 300,000 ha in 1970 to 4.49 million ha in 2008 (Mohd Basri, 2009) indicates the economic importance of this plantation crop and the growing world demand for palm oil. Palm oil has recently overtaken soybean oil as the world's leading vegetable oil, with Europe and China at present the major markets. The average oil yield in Malaysia ranges from 3 to 4.3 tonnes of oil per hectare per year (Corley and Tinker, 2003), which is substantially below the predicted physiological maximum of 17 t/ha/year (Corley, 1983). In 2008, Malaysia accounts for 41% of world palm oil production and 47% of world exports, and also of the world's total production and exports of oils (11%) and fats (25%) (MPOC, 2009).

1.2 Oil palm tissue culture and genetic transformation

Malaysian palm oil industry is faced with several challenges; lack of manpower resources and decrease in land availability for further expansion and also competition from other oils such as soybean oil, rapeseed oil and sunflower seed oil. Due to the increasing demand and land limitation, it is important to increase the yield and quality of palm oil faster than that has been achieved by conventional breeding. Thus, oil palm breeding is an extremely slow process due to the long generation period, time consuming and laborious. Innovative methods are needed to enhance the incorporation of new genetic resources into oil palm. Initially, tissue culture techniques were used to propagate elite oil palm clones (Jones, 1974). Tissue culture has made it possible to clonally propagate palms and to mass produce uniform planting material. The tissue culture technique for oil palm was developed in the 1970's by Staritsky and Rabechault et al., a decade after it was well established for other crops. With the development of

tissue culture it also opens new avenues for producing novel planting materials via genetic engineering, because tissue culture is the means for regeneration of tissues transformed with genes for traits of interest. A reliable transformation and regeneration systems is a pre-requisite for genetic engineering. It was identified as a powerful tool which complemented the efforts in traditional breeding (Puonti-Kaerlas et al., 1999). Currently, transgenic oil palms with novel traits such as high oleic acid, high stearic acid, ricinoleic acid and palmitoleic acid and synthesizing biodegradable plastics (polyhydroxybutyrate) were developed on a laboratory scale basis (Parveez, 2003).

1.3 Selectable markers

Plant transformation technology usually relies on the use of selectable marker genes, which are co-introduced with the gene of interest to distinguish, transformed from non-transformed cells. Selectable marker genes enable transgenic cells expressing the marker gene to survive in the presence of the appropriate selective agent. For the transformation of oil palm, the most common selection systems based on the use of the *npt*II gene encoding for neomycin phosphotransferase, thus conferring resistance to kanamycin and related aminoglycoside antibiotics such as paromomycin and geneticin (G418) and hygromycin phosphotransferase (*hpt*) gene, which confers resistance to hygromycin and the *bar* (bialaphos resistance) gene which confer resistance to the herbicide Basta^{TM} were tested as selection agents to inhibit growth of untransformed cells. Hygromycin and Basta^{TM} were found to be most suitable as selection agents for oil palm (Parveez et al., 1996). Production of transgenic oil palm resistant to herbicide Basta^{TM} was later established (Parveez, 1998).

1.3.1 Positive selection

The avoidance of antibiotic or herbicide resistance markers in genetically modified organisms is being practiced in certain laboratories. Recently, the use of antibiotic resistant genes were no longer authorised in the EU (European directive 2001/18/EC) neither for basic research (from January 1, 2009), nor approval of applications for field trials (as from January 1, 2005). Herbicide resistance itself can sometimes face acceptance problem and thus is not considered to be a preferred selection means. In this context, alternative selection systems for the recovery of transgenic plants have been developed. Among the available genes for positive selection, the *E. coli pmi* gene encoding a phosphomannose isomerase has been proven to be efficient for the recovery of transgenic plants (Bojsen et al., 1998, US Patent 5767378). Mannose is toxic for plant cells and is converted to mannose-6-phosphate, an inhibitor of glycolysis. This PMI/mannose selection system is based on the ability of transformed cells that have phosphomannose isomerase activity to convert mannose-6-phosphate into fructose-6-phosphate, an intermediate in the glycolysis pathway. 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, maize (Wright et al., 2001), rice (Lucca et al., 2001), 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). Moreover, the PMI protein is readily digestible in a simulated gastric environment, and revealed no adverse effects in an acute mouse oral toxicity study (Privalle, 2002), indicating a low allergenic potential. Therefore, the *pmi* gene appears to be an ideal selectable marker for plant transformation.

1.4 Aim of the thesis

A positive selection system based on the *pmi* gene as the selectable marker gene and mannose as selection agent was tested in oil palm. The main objective of this study is to develop a transformation system using mannose as a selection agent.

Strategy:

1. To construct the transformation vectors carrying *pmi* gene, driven by CaMV*35S* and *Ubi1* promoter.
2. To determine the optimal concentration of mannose for oil palm embryogenic calli.
3. To perform molecular and protein analyses to confirm the transgenic status of the transformed cultures and plantlets.

CHAPTER 2

LITERATURE REVIEW

2.1 Selectable marker genes for plant transformation

The production of transgenic plants with novel traits has relied largely on the use of selectable marker genes. The use of marker gene in a transformation process aims at giving a selective advantage to the transformed cells, allowing only them to grow faster and better, and killing the non-transformed cells (Brasileiro and Dusi, 1999). These are generally used in the initial stages of transformation for an early selection of transgenic cells (Sawahel, 1994). During the following regeneration steps, the influence exerted by the death of non-transformed cells on the transformed cells should be minimal on the selective medium. In most cases, the expression of selection marker genes is under the control of a constitutive promoter such as CaMV*35S*, nopaline synthase (*Nos*) or ubiquitin (*Ubi1*).

Approximately 50 selectable marker genes being used or being developed in transgenic plant research have been assessed for efficiency, biosafety, scientific applications and commercialization (Miki and McHugh, 2004). Selectable markers identified today can be differentiated into two types that enable transgenic plants or cells to be identified after transformation. They can be divided into positive and negative markers conferring a selective advantage or disadvantage, respectively.

2.1.1 Negative selectable marker genes

Negative selectable markers allow the selection of transformed cells or tissue explants by their ability to grow in the presence of negative selection pressure. There are two main categories of genes rising negative selection; the antibiotic and herbicide resistant genes (Miki and McHugh, 2004). Moreover, the presence of an antibiotic resistant gene in ingested plants is a matter of concern. In addition, selection of cells or tissues using negative selection requires precise timing of expression of the introduced genes in relation to the selection process. If the transgenic cells are treated with a toxic compound before the detoxifying gene is expressed or before enough gene product is produced to ameliorate the action of the toxic compound, both the transgenic and the non-transgenic cells are killed. If selection is delayed, the selection of transgenic cells or tissues may be hindered by, for example, shoot or callus formed from non-transgenic cells or tissues. The shoot or calli could form a barrier to the penetration of the compound used to select the transformed cells.

A number of dominant genes encoding enzymes rendering the plants resistant to antibiotics or herbicides are widely used as selectable makers in plant transformation (Table 2.1) (Miki and McHugh, 2004). Although many selectable markers are available, only a few have been used extensively in oil palm biotechnology. They include the genes *nptII* (Neomycin phosphotransferase II) and *hpt* (Hygromycin phosphotransferase) conferring antibiotic resistance and the herbicide tolerance gene *bar/pat* (Phosphinothricin acetyltransferase).

2.1.1.1 Antibiotic resistant marker genes

Antibiotic resistant marker genes confer the trait of resistance to a specific antibiotic. These genes correspond to the first genes successfully used to select transgenic plants.

Neomycin phosphotransferase II (nptII). The *nptII (*or *neo)* gene was isolated from the transposon Tn5 of *Escherichia coli* and it encodes NPT II (E.C.2.7.1.95), also known as aminoglycoside 3' phosphotransferase II (Fraley et al., 1983; Herrera-Estrella et al., 1983; Bevan et al., 1983). Aminoglycoside antibiotics that contain the 3'-hydroxyl group, such as the kanamycin A, B and C, neomycin, paromomycin and geneticin (G-418), are substrates for NPT II (Norelli and Aldwinckle, 1993). The *nptII* is the selective marker gene and kanamycin is the antibiotic most frequently used as selectable marker in transformation of a variety of crop plants, including dicots and monocots.

Hygromycin phosphotransferase (hpt). The *hpt* gene was isolated from *E.coli* and confers resistance to the hygromycin B antibiotic (Van den Elzen et al., 1985). It has been extensively utilized, especially when the use the *nptII* gene is not possible. This is the case of several monocotyledonous species that show high levels of natural resistance to kanamycin (Wilmink and Dons, 1993; Vasil, 1994). Hygromycin B is usually more toxic than kanamycin and kills sensitive cells more quickly.

2.1.1.2 Herbicide tolerant marker genes

Herbicide tolerant marker genes confer an agronomic characteristic, tolerance to the application of a specific herbicide. For instance, the gene conferring tolerance to the herbicide glufosinate ammonium is often used as a selectable marker in plant biotechnology. Those cells which survive the exposure to the herbicide are selected and regenerated into whole organisms.

Phosphinothricin N-acetyltransferase (*PAT*) (pat and bar). The *pat* and *bar* genes were isolated from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*, respectively. The gene encodes the enzyme PAT. They have been extensively used as selectable marker in plants (Murakami et al., 1986). The PAT enzyme inactivates herbicides with phosphinothricin (PPT) as active compound, such as Basta™, Liberty™ and Herbicide™. The *bar* gene is found in strains of *S. hygroscopicus* that produce bialaphos, a tri-peptide antibiotic that consists of PPT and two L-alanine residues (Murakami et al., 1986). The *bar* gene (PAT) product protects these strains from the action of its own antibiotic, metabolizing PPT into inactive acetylated derived compound and preventing auto toxicity (De Block et al., 1987; Mazur and Falco, 1989). The *bar* gene is the most widely and successfully used selectable marker gene for all of the major cereal species such as wheat, rice, maize, barley, sorghum, oats and rye (Vasil, 1994; Vain et al., 1995).

Table 2.1: Antibiotics and herbicides used as selection agent in plant transformation.

Marker gene	Enzyme encoded	Selective agent	Reference
Antibiotics			
nptII	Neomycin phosphotransferase II	Kanamycin Neomycin Geneticin (G418)	Bevan et al., 1983 Fraley et al., 1983 Herrera-Estrella et al., 1983
hph, hpt	Hygromycin phosphotransferase	Hygromycin B	Waldron et al., 1985 Van den Elzen et al., 1985
aacC3, aacC4	Gentamycin-3-*N*acetyltransferase	Gentamycin	Hayford et al., 1988
ble	not known	Bleomycin Phleomycin	Hille et al., 1986
dhfr	Dihydrofolate reductase	Methotrexate	Herrera-Estrella et al., 1983
SPT	Streptomycin phosphotransferase	Streptomycin	Jones et al., 1987
aadA	16S rRNA Aminoglycoside-3.-adenyltransferase	Spectinomycin Streptomycin	Svab et al.,1990a Svab et al., 1990b
Herbicides			
bar	Phosphinothricin acetytransferase	Phosphinothricin	De Block et al., 1987
als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazolinones	Haughn et al., 1988
aroA	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate (Roundup)	Comai et al., 1985 Shah et al., 1986

2.1.2 Positive selectable marker genes

Positive selection, a new concept for selection of transgenic plant cells, was developed later, where the selective agent is converted into fully metabolised compounds (Joersbo and Okkels 1996; Haldrup et al., 1998a, b; Joersbo et al., 1998; Joersbo, 2001). The use of this new mode of selection has been demonstrated to be successful in a large variety of monocotyledonous and dicotyledonous species. This selection differs from more traditional modes of selection in which compounds such as antibiotics or herbicides are used to kill non-transformed cells (negative selection). In the case of positive selection, a transformed cell acquires the ability to metabolize a substrate that it previously could not use or not use efficiently and thereby grows out of the mass of non-transformed tissue. The transgenic cells are selected by addition of a compound which is converted by the transformed cells into a compound inducing a positive response, such as growth or shoot formation. Non-transgenic cells stay alive without shoot formation, which means that neighbouring cells are not exposed to toxic secretions from dying cells. Simultaneously, cells containing the transgene can utilize a component in the medium which results in growth or differentiation, and non-transformed cells remain unaffected, therefore having no detrimental effect on transgenic cells.

Positive selection can be of many types from inactive forms of plant growth regulators that are then converted to active forms by the transferred enzyme to alternative carbohydrate sources that are not utilized efficiently by the non-transformed cells that become available upon transformation with an enzyme that allows them to be metabolized. Non-transformed cells either grow slowly in comparison to transformed cells or not at all. Using positive selection, non-transformed cells may die, but, typically, production of phenolic compounds observed with negative selection markers does not occur. In many cases, this effect contributes to higher transformation

efficiencies, as these compounds can negatively influence the growth of transformed cells. Positive selection systems using either mannose (Joersbo et al., 1998, 1999), xylose (Haldrup et al., 1998a, b) or deoxyglucose (Kunze et al., 2001), which do not cause any risk to animal, human or environmental safety, have been shown to be more efficient for potato, tomato, tobacco and sugar beet transformation than methods based on antibiotic selection. Most of these markers have been developed in model plants, e.g. tobacco or *Arabidopsis*. The positive selectable marker gene on *DOG^{R}1* (2-Deoxyglucose-6-phosphate phosphatases) was evaluated in oil palm (Dayang Izawati, 2009).

2.1.2.1 Phosphomannose isomerase (PMI)

Phosphomannose isomerase (PMI, EC 5.3.1.8) is common in nature and found in bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) and mammals (*Amophophallus konjac*), as well as humans (*Homo sapiens*) (Slein, 1950; Gracy and Noltmann, 1968; Murata, 1975; Miles and Guest, 1984; Darzins et al., 1986; Shinabarger et al., 1991; Collins and Hackett, 1991; Schmidt et al., 1992; Smith et al., 1992; Proudfoot et al., 1994). However, PMI is less ubiquitous in plants. It has been reported to be present in only a few species like soybeans and several other legumes but absent in many plants (Goldsworthy and Street, 1965; Lee and Matheson, 1984; Privalle et al., 2000).

The selection system employing the *manA* gene is highly versatile. The gene code for PMI was cloned from *E.coli* (Miles and Guest, 1984). PMI is a necessary enzyme of mannose metabolism in vascular plants. It catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate (Figure 2.1). In plants, the six carbon aldehyde mannose is an intermediate in the pathway of mannitol metabolism (Stoop et

al., 1996), may be a precursor to ascorbate synthesis (Wheeler et al., 1998), is a critical component of cell wall polymers (Olczak and Watorek, 2000), and is also a component of oligosaccharide modifications made to glycoproteins including cell membrane receptors (Strahl-Bolsinger et al., 1999). In the presence of mannose in transformed cells, the PMI converts mannose-6-phosphate into fructose-6-phosphate (Figure 2.2). The product can be immediately incorporated in the plant metabolic pathway (Privalle et al., 2000). Thus, the mannose can be used as sole source of carbohydrate in the transformed cells. This selection system is immediate and extremely efficient (Joersbo et al., 1998). 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 could minimize the plant growth due to mannose-6-phosphate accumulation. Even though most of the plant species are sensitive to mannose, some species, especially dicotyledonous including carrot, tobacco, sweet potato and leguminous crops have shown a considerable insensibility to this sugar. Other species are extremely sensitive and were successfully transformed using mannose as selective agent such as sugarbeet (Joersbo et al., 1998), maize (Negrotto et al., 2000; Wang et al., 2000), wheat (Wright et al., 2001), rice (Lucca et al., 2001), sweet orange (Boscariol et al., 2003), oat, barley, tomato, potato, sunflower, oil seed rape and pea (Joersbo et al., 1999, 2000).

The principle of this approach is the inability of some plants to use mannose as carbon source. The major difference from selection based on antibiotics or herbicides, which kill the non-transformed cells, where as the mannose selection system arrests the growth and development of non-transformed cells by carbohydrate starvation (Wang et al., 2000) but still survive (Haldrup et al., 1998a, b). Owing to this growth advantage of transformed cells, this strategy is called ‘positive selection’ (Joersbo and Okkels, 1996).

Table 2.2: Positive selection of transgenic plants.

Selection Agents	Genes	Enzymes	Sources	Genome	References
Positive selection of transgenic plants use toxic drugs, metabolite analogues and enzymes					
2-Deoxyglucose	*DOG^{R}1*	2-Deoxyglucose-6-phosphate phosphatase	*Saccharomyces cerevisiae*	Nuclear	Kunze et al.,2001
Betaine aldehyde	*BADH*	Betaine aldehyde dehydrogenase	*Spinacia oleracea*	Nuclear, plastid	Ursin, 1996; Daniell et al., 2001
S-Aminoethyl L-cysteine (AEC)	*DHPS* *ocs*	Dihydropicolinate synthase Octopine synthase	*Escherichia coli* *Agrobacterium tumefaciens*	Nuclear	Perl et al., 1993; Koziel et al., 1984
4-Methyltryptophan (4-mT)	TDC	Tryptophan decarboxylase	*Catharanthus roseus*	Nuclear	Goddijin et al., 1993
Methotrexate	DHFR	Dihydrofolate reductase	*Escherichia coli*	Nuclear	Herrera-Estella et al.,1983;Eichholtz et al.,1987
			Candida albicans	Nuclear	Irdani et al., 1998
Positive selection of transgenic plants use non-toxic agents and enzymes					
D-Xylose	xylA	Xylose isomerase	*Streptomyces rubignosus*	Nuclear	Haldrup et al., 1998a;
			Thermoanaerobacterium Sulfurogenes		Haldrup et al., 1998b
D-Mannose	***manA* (*pmi*)**	**Phosphomannose isomerase**	***Escherichia coli***	**Nuclear**	**Joersbo et al., 1998**
Benzyladenine-N	*uidA*	• -Glucuronides -3-glucuronide (*gusA*)	*Escherichia coli*	Nuclear	Joersbo and Okkel, 1996

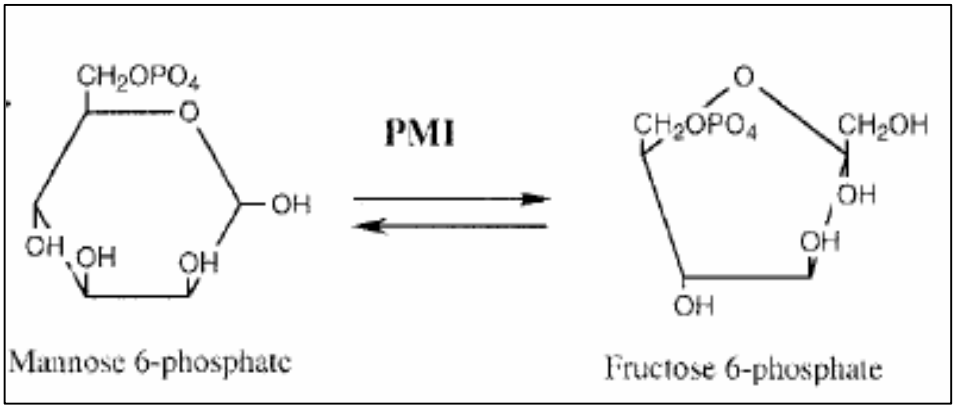

Figure 2.1: Reaction catalyzed by phosphomannose isomerase (Reed et al., 2001).

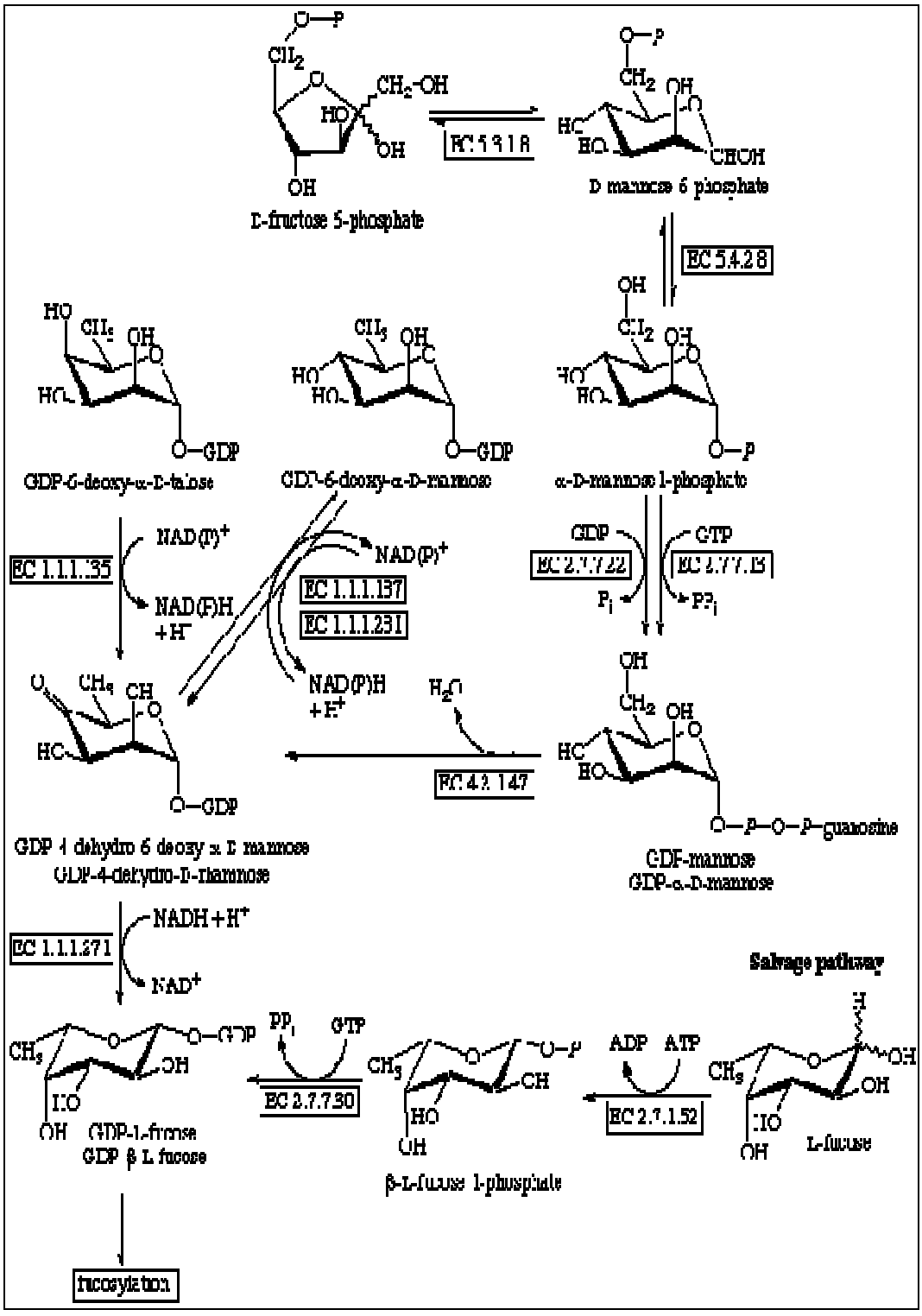

Figure 2.2: Phosphomannose isomerase pathway.
Source: http://www.chem.qmul.ac.uk/iubmb/enzyme/EC5/3/1/8.html

Advantages of* PMI *system. Positive selection of transgenic plant cells using mannose has several advantages in plant transformation. Firstly, the transformation frequencies obtained by positive selection appear to be higher than those obtained with antibiotic or herbicide selection (Joersbo et al., 1998; Haldrup et al., 1998a, b). Secondly, it allows avoiding the use of antibiotic or herbicide resistance marker genes, which cause widespread public concern because of inadequate knowledge of the agent's impact on the environment and on human health (Ferber, 1999). Thirdly, it does not cause any risk to animal, human or environmental safety (Joersbo et al., 1998; Haldrup et al., 1998a, b; Negrotto et al., 2000; Wang et al., 2000).

2.1.2.1.1 Mannose and its mode of action

Mannose, a hexose sugar has been known for a number of years to be unable to sustain growth of various plant species (Malca et al., 1967). Mannose and mannose derivatives are common constituents of living cells and are key components of intermediary metabolism (Figure 2.3). Mannose cannot usually be metabolized by non-transformed cells and is converted into mannose-6-phosphate by endogenous hexokinase. This impact actually results from the accumulation of mannose-6-phosphate, which inhibits phosphoglucose isomerase, thus blocking glycolysis (Goldsworthy and Street, 1965). Other impacts include depletion of the pyrophosphate required for ATP production (Goldsworthy and Street, 1965; Herold and Lewis, 1977) and transcriptional repression of genes associated with photosynthesis and glyoxylate cycle (Jang and Sheen, 1994, 1997; Graham et al., 1997).

Using traditional transformation protocols, plant cells are placed on culture media containing salts, hormones and a carbon source, usually sucrose. For the PMI/mannose selection system, plant tissues are cultured on a similar medium supplemented with

either mannose as the unique source of carbon or with media containing both sucrose and mannose. While mannose has no direct adverse effect on plant cells, the subsequent selection is considered to be a consequence of its phosphorylation to mannose-6-phosphate by hexokinase. Thus, when mannose is added to the culture medium, plant growth may be minimized due to mannose-6-phosphate accumulation. The mannose-6-phosphate toxicity in plant cells was shown to be responsible for apoptosis, or programmed cellular death, through induction of an endonuclease, responsible for DNA laddering (Stein and Hansen, 1999). In the presence of PMI, mannose-6-phosphate enters the glycolytic pathway after isomerization to fructose-6-phosphate (Appendix F). Mannose is also the major sugar residue in the carbohydrate portion of glycoproteins. Recently mannose has been identified as a precursor for ascorbate synthesis (Wheeler et al., 1998). Therefore, mannose is a hexose that fills the desirable requirements for a good selection agent; it is soluble in plant culture media, absorbed by plant cells, cheap, easily available and safe, and the transformation efficiencies are normally higher when compared to herbicide or antibiotic resistance genes (Aragao and Brasileiro, 2002).

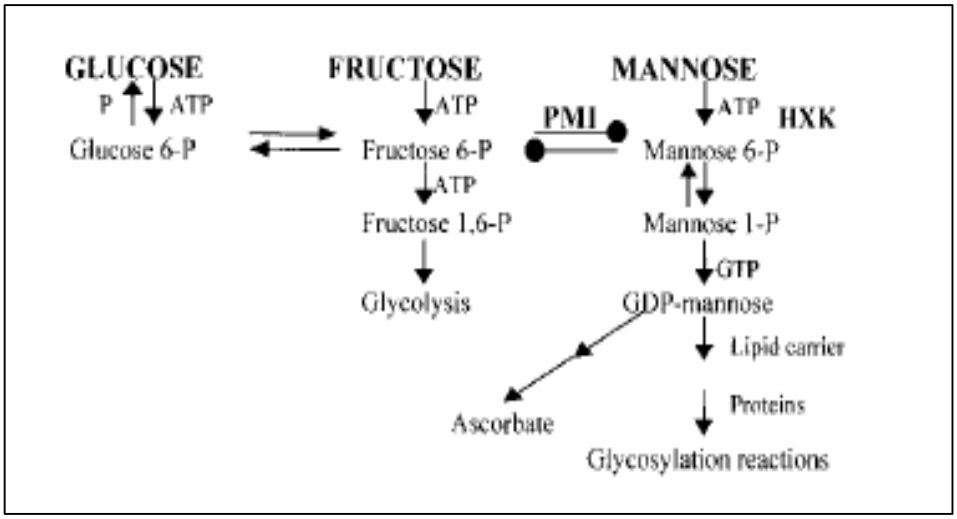

Figure 2.3: Basic intermediary metabolism involving mannose in nonleguminous plant cells not transformed with PMI. The reaction catalyzed by PMI is indicated by the solid lines with rounded arrows (Privalle et al., 2002).

2.1.2.1.2 Principle of PMI selection in transgenic plants

PMI has been utilized as a selectable marker for transformation of many plant species as shown in table 2.3. Plant cells are genetically transformed to express PMI acquire a growth advantage (positive selection) on mannose-containing media. They are protected both from the negative impact of mannose derivatives and can utilize the mannose as a carbon source. Hence with the *pmi* gene, mannose is a useful selectable agent for the generation of transgenic plants (Hansen and Wright, 1999). Plant cells take up mannose and convert it to mannose-6-phosphate, an inhibitor of glycolysis. The production of mannose-6-phosphate also depletes the cell of inorganic phosphate. PMI activity converts mannose-6-phosphate to fructose-6-phosphate, an intermediate of glycolysis that positively supports growth, improving the energy status of the transformed cells and avoiding accumulation of derivatized selective agents.

The non-transgenic PMI negative cells are unable to survive on media containing mannose as the sole carbon source. Mannose will be converted into mannose-6-phosphate by endogenous hexokinase (Ferguson et al., 1958; Malca et al., 1967). Mannose-6-phosphate is not further utilized due to a deficiency of phosphomannose isomerase (PMI). The synthesis of mannose-6-phosphate depletes cells of orthophosphate that is required for ATP production. The ATP as well as phosphate starvation deplete cells of energy for critical functions such as cell division and elongation, resulting in severe growth inhibition (Goldsworthy and Street, 1965; Loughman, 1966; Joersbo et al., 1998).

Joersbo et al., 1998 observed that the use of mannose selection in sugar beet (*Beta vulgaris* L.) resulted in a ten-fold increase in transformation frequency when compared with the frequencies obtained using the *nptII* gene and kanamycin as the selective agent.

These results were further supported by similar findings in maize, wheat, barley, watermelon (Reed et al., 2001) and in rice (Lucca et al., 2001). 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 the high transformation frequencies reported. Only in a few species, such as cassava, the frequency of transformation was lower than that achieved with the *hpt* gene (Zhang and Puonti-Kaerlas, 2000).

The system is being marketed as the Positech^{TM} selection technique by Syngenta. Safety assessments have been performed including the allergenicity and toxicity studies (Privalle et al., 2000; Reed et al., 2001; Privalle, 2002). The enzyme was found to be completely digested in simulated mammalian gastric and intestinal fluids. PMI protein showed no adverse effects on mice following acute oral toxicity studies. Furthermore, there appeared to be no changes in the glycoprotein profiles of transgenic maize or sugar beets. Field trials conducted on seven independent transformation events demonstrated that there were no differences in the agronomic performance or grain composition of transgenic maize compared to non-transgenic controls (Privalle et al., 2000; Reed et al., 2001).

Table 2.3: List of plants transformed using PMI as selectable marker gene.

Plant	Transformation Method	Reference
Tomato	*Agrobacterium*	Briza et al., 2008; Sigareva et al., 2004
Potato	*Agrobacterium*	Briza et al., 2008
Chinese cabbage	*Agrobacterium*	Min et al., 2007; Ku et al., 2006
Torenia	*Agrobacterium*	Li et al., 2007
Flax	*Agrobacterium*	Lamblin et al., 2007
Cucumber	*Agrobacterium*	He et al., 2006
Sugarcane	Biolistic	Jain et al., 2007
Almond	*Agrobacterium*	Ramesh et al., 2006
Onion	*Agrobacterium* Biolistic	Aswath et al., 2006
Apple	*Agrobacterium*	Degenhardt et al., 2006
Sorghum	*Agrobacterium*	Gao et al., 2005; Songul et al., 2009
Papaya	Biolistic	Zhu et al., 2005
Bentgrass	*Agrobacterium*	Fu et al., 2005
Durum wheat	Biolistic	Gadaleta et al., 2004
Pearl millet	Biolistic	O' Kennedy et al., 2004
Sweet orange	*Agrobacterium*	Boscariol et al., 2003
Hemp	*Agrobacterium*	Feeney and Punja, 2003
Pepper	*Agrobacterium*	Kim et al., 2002
Rice	*Agrobacterium*	Lucca et al., 2001; Datta et al., 2003; He et al., 2004, Zai-Song et al., 2006
Cassava	Biolistic	Zhang et al., 2000
Maize	Biolistic *Agrobacterium*	Wright et al., 2001 Negrotto et al., 2000; Evans et al., 1996 Wang et al., 2000; Reed et al., 2001
Wheat	Biolistic	Wright et al., 2001; Reed et al., 2001
Sugar beet	*Agrobacterium*	Joersbo et al., 1998; 2000
Arabidopsis	*Agrobacterium*	Melanson et al., 1999; Todd and Tague, 2001

2.1.2.2 *2-Deoxyglucose-6-phosphate phosphatases*

The glucose analogue, 2-deoxyglucose (*2-DOG*), is phosphorylated by hexokinase to form 2-DOG-6-phosphate. 2-DOG-6-phosphate competes with glucose-6-phosphate causing cell death through the inhibition of glycolysis. *DOG*^{R}*1* gene, which has been isolated from yeast, gives resistance to 2-deoxyglucose *(2-DOG)* when over-expressed in transgenic plants. The selection system was demonstrated in pea (Sonnewald and Ebneth, 1998), tobacco and potato plants (Kunze et al., 2001). Abnormalities were not observed in the plants presumably due to the narrow substrate specificity of the enzyme (Kunze et al., 2001).

2.1.2.3 *Xylose isomerase*

*X*ylose isomerase (*xylA*) gene isolated from *Thermoanaerobacterium thermosulfurogenes* or from *Streptomyces rubiginosus* is employed as the selectable marker and xylose as the selective agent (Haldrup et al., 1998a, b). The enzyme from *S. rubiginosus* catalyses the isomerization of D-xylose to D-xylulose. The non-transformed plant cells cannot utilize the D-xylose as a sole carbon source. But cells which are transformed with *xylA* growing on xylose have the ability to convert D-xylose to D-xylulose and utilize it as a carbon source. Transgenic plants of potato, tobacco and tomato were successfully selected in xylose containing media (Haldrup et al., 1998a, b).

2.2 Non-selectable maker gene systems: reporter genes

2.2.1 •-*Glucuronidase gene*

The enzyme •-glucuronidase (GUS, E.C. 3.2.1.31) (Jefferson, 1987), encoding by the *E. coli uidA* (*gusA*) gene. GUS catalyses the hydrolysis of •-D-glucuronides. The glucuronide substrate has been conjugated with the cytokinin, benzyladenine, to create benzyladenine *N*-3-glucuronide which does not affect plant growth and differentiation.

However, hydrolysis by GUS releases benzyladenine which will stimulate shoot regeneration. This process has been shown to be an effective conditional-positive selection strategy in tobacco (Joersbo and Okkels, 1996). The activity of *gus* gene is easily detected histochemically by using X-Gluc as substrate which results in blue colour spot. It can also be detected fluorometrically using MUG as substrate. Although the assay is destructive, this reporter gene is widely used (Parveez, 1998).

2.2.2 *Luciferase gene*

The luciferase reporter gene (*luc*) (Ow et al., 1986), isolated from *Photinus pyralis*, was used for selection of transgenic plants in *Dendrobium* (Chia et al., 1994) and cassava (Raemakers et al., 1996). Although the luciferase assay is expensive and labor intensive, its nondestructive nature has advantages over the GUS assay. As a reporter, luciferase (LUC) can be monitored in living tissue but this requires specialized detection equipment (Ow et al., 1986).

2.2.3 *Green fluorescent protein gene*

The green fluorescent protein (*gfp*) gene was isolated from jellyfish, *Aequorea victoria* (Chalfie et al., 1994). The *gfp* gene encodes a small, barrel-shaped protein surrounding a fluorescent chromophore, which immediately emits green fluorescent light in the blue to ultraviolet range. Visual detection is possible at any time in living cells without their destruction and the addition of any cofactor or external substrate. In addition, *gfp* gene product does not adversely affect cell growth, regeneration and fertility of transformed plants. GFP selection system seems to be promising for tissue culture/transformation systems that are inefficient, recalcitrant genotypes, and plant species for which no system exists. It can be used to monitor gene expression and protein localization in

living cells and does not interfere with normal cell growth or function (Parveez and Na'imatulapidah, 2008).

2.3 Plant transformation

Plant transformation is now a core research tool in plant biology and a practical tool for cultivar improvement, especially those that are difficult to modify through conventional breeding methods. The genetic transformation of plants is the process where a defined fragment of DNA is introduced and integrated into the genome of the plant. Three prerequisites to apply genetic transformation for plant improvement are needed. First is a reliable regeneration system which is compatible with transformation methods allowing regeneration of transgenic plants. The second requirement is, an efficient way to introduce DNA into the regenerable cells. Lastly, a procedure to select and regenerate transformed plants at a satisfactory frequency is also required (Birch, 1997).

2.3.1 Plant regeneration *in-vitro*

In plant regeneration systems for transformation, the most important factor is a source of large number of regenerable cells which are accessible to the gene transfer treatment, and will retain their capacity for regeneration during target preparation, cell proliferation and selection treatments. Plant regeneration through tissue culture has made it possible to clonally propagate palms and mass produce uniform planting materials. Plant cell and tissue culture has become a major tool in the study of an increasing number of fundamental and applied programs in plant science. Tissue culture techniques are being used globally for the exist conservation of plants. Tissue culture approach is an efficient method for rapid *in vitro* clonal propagation of transgenic plants. Tissue culture is often referred to as the art and science of growing plant parts into whole plants. This is achieved under aseptic conditions *in vitro* or 'in glass' (George, 1993). Examples of the

different plant parts (explants) used in tissue culture are; plant culture (the culture of seedlings or larger plants), embryo culture (the culture of isolated mature or immature embryos), callus culture (the culture of tissue arising from explants of plant organs), suspension or cell culture (the culture of isolated cells or aggregates in a liquid medium) and protoplast culture (the culture cells devoid of their cell wall).

2.3.2 Gene transfer techniques

Most commonly used systems to transfer foreign genes into plant cells are particle bombardment and *Agrobacterium*-mediated gene transfer. Other gene transfer methods, such as protoplast transformation (Paszkowski et al., 1984; Shillito 1999), microinjecting DNA into cells or zygotes (Schnorf et al., 1991; Leduc et al., 1996), silicon carbide whisker-mediated DNA transfer (Thompson et al., 1995) and electroporation (Fromm et al., 1986; Lurquin, 1997), have also been developed in plant transformation. However, the transformation efficiency of these techniques is low.

2.3.2.1 *Agrobacterium*-mediated transformation

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become one of the nature's most successful plant genetic engineer. The plant pathogen has the ability to precisely transfer defined DNA sequences to plant cells which has been very effectively utilized in the design of a range of Ti (tumour inducing) plasmid-based vectors. The current status of understanding of *Agrobacterium* T-DNA transfer process has been reviewed by Gelvin (2003). Three genetic elements, *Agrobacterium* chromosomal virulence genes (*chv*), T-DNA delimited by a right border and a left border and Ti plasmid virulence genes (*vir*) constitute the T-DNA transfer machinery. Important events of T-DNA transfer and components involved in the process are outlined in Figure 2.4. Unfortunately, until recently, the hosts for

Agrobacterium have been limited to dicotyledoneous plants (Zhao et al., 2006a; Permyakova et al., 2009; Bhatti and He, 2009) and a few monocotyledonous plants such as rice (Cheng et al., 1998), wheat (Hu et al., 2003), sorghum (Carvalho et al., 2004), banana (Zhao et al., 2006b) and barley (Shrawat et al., 2007). For long term measure, the inability of *Agrobacterium* to transfer DNA to monocotyledonous plants was considered as its major limitation. *Agrobacterium*-mediated transformation has remarkable advantages over direct transformation methods. It reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppresion and instability (Koncz et al., 1994, Hansen et al., 1997). In addition, it is a single-cell transformation system and not forming mosaic plants, which are more frequent when direct transformation is used (Enríquez-Obregón et al., 1997, 1998).

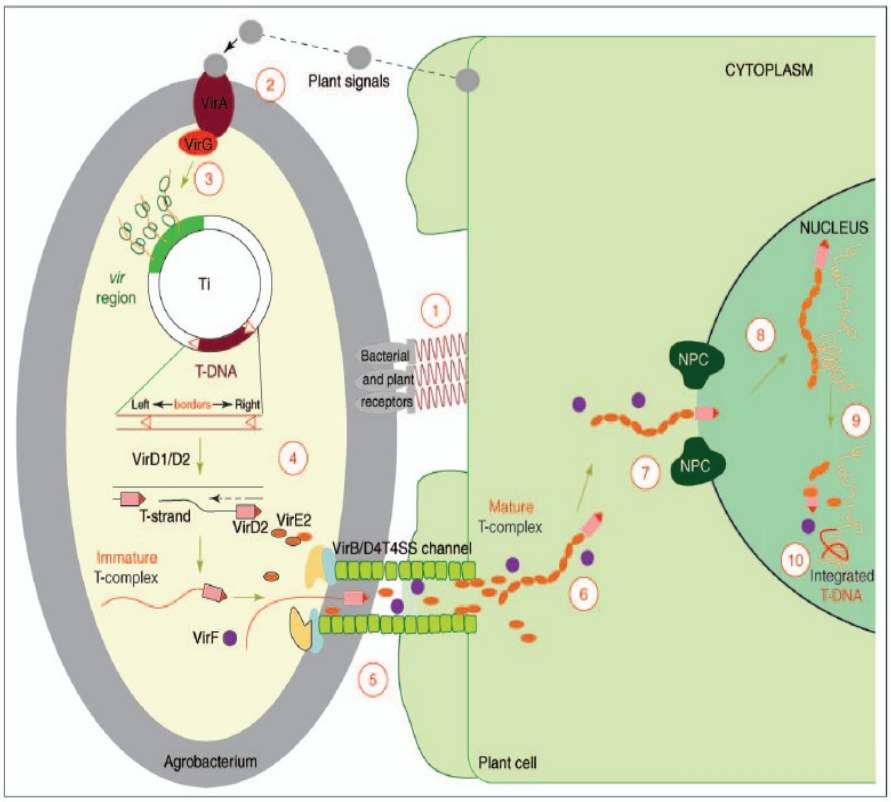

Figure 2.4: A model for the *Agrobacterium*-mediated genetic transformation depicting the cascade of events leading to T-DNA transfer from *Agrobacterium* to the plant genome (Tzfira and Citovsky, 2000).

2.3.2.2 Biolistic transformation

Methods of direct gene transfer avoiding the host range limitations of *Agrobacterium* have been developed as well. Sanford and co-workers established a method for gene delivery by microprojectile bombardment (Klein et al., 1987). Microprojectile bombardment mediated transformation (Figure 2.5) is one of the most promising gene transfer techniques even for those plants which have proven recalcitrant to transformation by any other procedure. DNA-coated microprojectiles are accelerated by means of an explosion or a burst of gas to a sufficient velocity to penetrate plant cell walls and release the DNA for expression and/or integration in the plant genome. Finer et al., (1992) developed the system further and constructed the Particle Inflow Gun (PIG), which is inexpensive and simple to use. Biolistics has now become the second most widely used technique for plant transformation after *Agrobacterium*-mediated transformation. This technique has been successfully used to transform meristems and tissues with high regeneration potential, to deliver foreign DNA into intact and regenerable cells, tissues and organs with no limitation of *Agrobacterium* related host specificity and tissue culture related regeneration difficulties. It has also been used to transform organelles such as chloroplasts (Luthra et al., 1997; Klein and Jones, 1999). Many important crop plants, for example, rice (Ke et al., 2001), maize (Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992, 1993), soybean (McCabe et al., 1988; Finer and McMullen, 1991), cassava (Shöpke et al., 1996, Raemakers et al., 1996) and oil palm (Parveez, 1998) have been successfully transformed using biolistic.

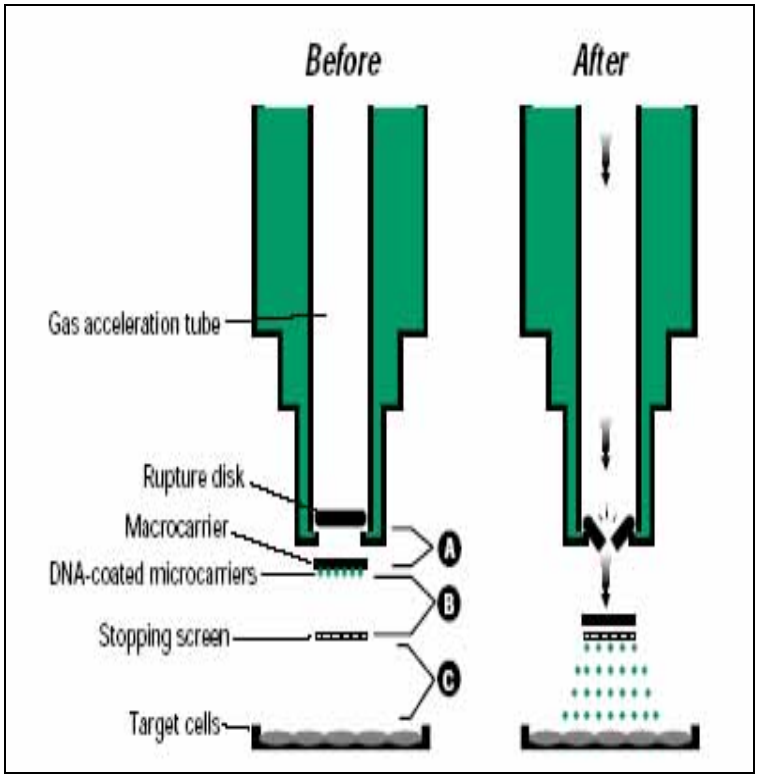

Figure 2.5: Schematic representation of the operation of microprojectile bombardment (Adapted from Heiser, Bio-Rad Bulletin1688EG).

2.4 Genetic engineering of oil palm

Genetic engineering of oil palm has high potential as an efficient complement to traditional breeding in improving agriculturally valuable traits such as disease resistance and herbicide resistance. This could be due to the long generation period and requirement of large areas for breeding (Rajanaidu et al., 1993; Sambanthamurthi et al., 2000). Furthermore, genetic engineering allows modifications to be carried out with higher precision compared to breeding. With such advantages, application of genetic engineering for oil palm improvement is believed to be able to enhance the competitiveness and ensure sustainability of the industry (Basiron and Chan, 2005; Sambanthamurti et al., 2000).

The effort to carry out the genetic engineering work in oil palm is relatively recent. The program was only initiated in the late 80s. With the recent advancement of genetic engineering techniques, the effort to improve oil palm through genetic engineering was escalated in the 90s. Due to the high interest of high oleic oil, the main target of oil palm genetic program has been the modification of fatty acid biosynthesis pathway towards the production of high oleic oil. This has led to the vigorous efforts towards the biochemical studies and isolation of genes and promoters related to the oil palm fatty acid biosynthesis pathway. Other products targeted are high stearic acid, biodegradable plastic, pharmaceuticals and nutraceuticals such as lycopene and palmitoleic acid, and industrial oils such as ricinoleic acid (Parveez, 2003; Sambanthamurthi et al., 2000, Parveez et al., 1999). Nevertheless, further improvement of the genetic transformation method has been given a priority to ensure successful manipulation of oil palm in years to come. One of the approaches taken to improve the efficiency of oil palm genetic transformation is through the application of a novel selectable marker system. To date, regeneration of transgenic oil palm using herbicide Basta as negative selection has been established (Parveez, 2000). However, the potential consequences of antibiotic or herbicide resistant genes on the environment and on human health, especially in food crops, have raised widespread public concern (Wang et al., 2000). In response to this, alternative selection systems for the recovery of transgenic plants have been successfully developed using positive selectable markers (Haldrup et al., 1998a, b; Joersbo et al., 1998; Hansen and Wright, 1999) such as *pmi* gene has been developed in this study.

2.4.1 Tissue culture

For oil palm there are no *in vivo* (in life) methods available for vegetative propagation. Oil palm does not produce offshoots, as all the auxiliary buds form inflorescences and there are no existing methods for the establishment of cuttings. Tissue culture has made it possible to clonally propagate palms and mass produce uniform planting materials. The tissue culture technique for oil palm was developed in the 1970's, a decade after it was well established for other crops. The oil palm industry has been quick to capitalise and commercialise this technology. Figure 2.6 show the stages in oil palm tissue culture.

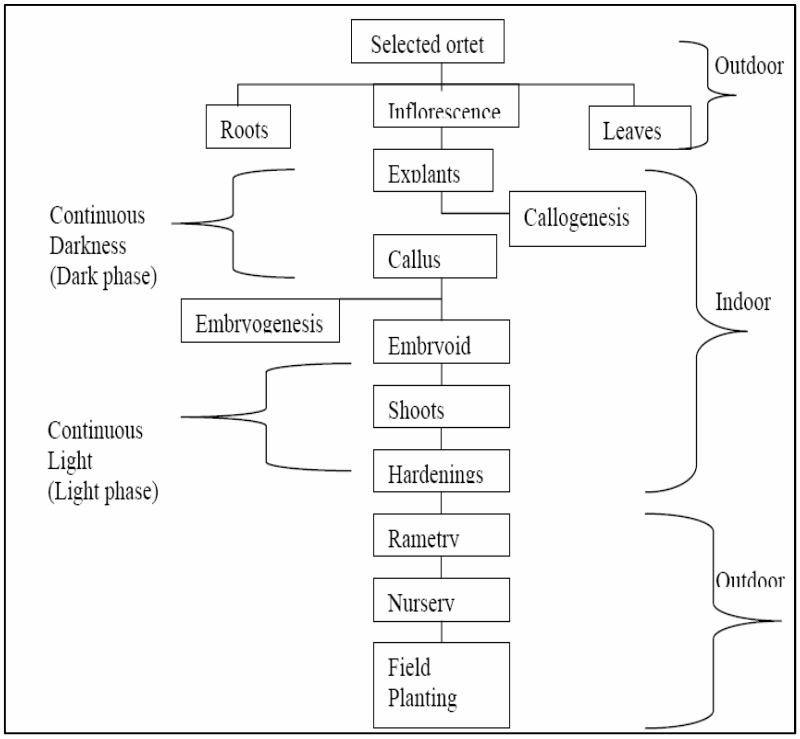

Figure 2.6: Stages in oil palm tissue culture (Lord and Vovola, 2004). The process start with the selection of ortets, from which callus is generated. Callus can be generated from a number of different tissue including leaves, inflorescences and roots can be used as explants. Callus is usually initiated from the explants by incubation in the dark at 28 ± 1^{o}C, followed by embryogenesis, shoot and root regeneration, hardening of ramets for the nursery and finally field evaluation.

Now, about 20 oil palm laboratories are in operation throughout the world with capacities ranging from 10,000 – 200,000 plantlets per year (Zamzuri et al., 1999). As compared to seed production, tissue culture of oil palm offers several advantages (Sogeke, 1998). It allows rapid multiplication of uniform planting materials with desired characteristics. This enables improvement of planting materials using existing individuals which have all or most of the desired qualities such as good oil yield and composition, slow vertical growth and disease resistance. Additionally, it also opens new avenues for producing novel planting materials via genetic engineering, because tissue culture is the means for regeneration of tissues transformed with genes for traits of interest.

2.4.2 Methods of oil palm transformation

Oil palm has been successfully transformed through 2 methods; biolistic and *Agrobacterium* mediated transformation. The biolistic process, first reported by Sanford et al., (1987), was initially chosen as a method for oil palm transformation as it has been the most successful method for transforming monocotyledons then (Parveez et al., 2000; Na'imatulapidah and Parveez, 2007). *Agrobacterium* mediated transformation of oil palm was also developed for oil palm (Abdullah et al., 2005; Dayang Izawati et al., 2008). The *Agrobacterium* mediated transformation has not been very successful in monocot as compared to its counterpart, dicots. This is primarily due to the naturally non-host of *Agrobacterium* for monocots. By comparing the *Agrobacterium* and biolistic methods in terms transformation rate in oil palm, Dayang Izawati et al., 2008 observed that the transformation rate was at 0.7% which was slightly lower than biolistic, 1-1.5%.

2.4.3 Potential applications of oil palm genetic engineering

In MPOB, the system developed has been used to transform oil palm with genes targeted for products of interest. High oleate transgenic oil palm was given the top prioritized product. The effort to produce high oleate transgenic oil palm has been carried out using two plant transformation constructs namely a construct containing an antisense copy of palmitoyl-ACP thioesterase gene and a construct containing a combination of antisense copy of palmitoyl ACP thioesterase and a sense copy of stearoyl ACP-desaturase. Transformed plantlets have been obtained and the presence of the *bar* and palmitoyl ACP-thioesterase genes has been confirmed by PCR analysis. However, the comprehensive data on the actual changes in the fatty acid composition have not been reported (Parveez et al., 2005).

The application of the system has also been extended into generating transgenic oil palms producing biodegradable plastic. The presence of target genes in the resulted transgenic plantlets was confirmed by PCR analysis (Parveez, 2003). A detectable amount of PHB was observed in some of the transgenic plantlets (Parveez et al., 2005, 2008).

Constructs containing an antisense copy of stearoyl-ACP desaturase have also been bombarded into oil palm calli for the production of high stearate transgenic plants. Putative transgenic plants carrying the gene have been obtained. However, no molecular or fatty acid analyses have been reported to date (Parveez, 2003).

2.4.4 Selectable markers and promoters in transgenic oil palm

Production of transgenic oil palm using embryogenic calli (Parveez, 2000) and utilization of immature embryos (Na'imatulapidah and Parveez, 2007) as target tissue have been established. Kanchanapoom et al., 2008 reported that embryogenic callus is the best target tissue for biolistic transformation of oil palm when compared to immature embryos and young seedlings, with a 100% transformation efficiency obtained. This was produced using the herbicide Basta™ and antibiotic hygromycin resistance genes as selectable markers after co-cultivation with *Agrobacterium* (Dayang Izawati et al., 2008) or particle bombardment (Parveez, 1998). Condition for delivering DNA into embryogenic calli has been optimized successfully based on transient *gusA* gene expression (Parveez et al., 1997, 1998).

Promoters are a critical part of constructs for transformation. While constitutive promoters are useful for selectable markers and some gene of interest, targeting gene expression to specific plant parts or at specific developmental times is more effective for many transgenes. Much of the transgenic research in oil palm has used the maize ubiquitin and cauliflower mosaic virus promoters for expression of transgenes throughout the plant and all its growth stages. *Emu*, *Ubi1*, actin, cauliflower mosaic virus (CaMV*35S*) and *Adh1* constitutive promoters were evaluated and significant effects on transient *gusA* gene expression were demonstrated by each of the different promoters tested (Chowdhury et al., 1997, Parveez, 1998). It was indicated that *Ubi1* and *Emu* were found to be the most efficient constitutive promoters to drive high expression of GUS reporter gene in oil palm embryogenic calli and young leaftlet from matured palm, respectively; followed by CaMV*35S*, *Act1* and *Adh1*. All studies indicated that the *Ubi1* was the most efficient for all monocotyledonous plants (Christensen and Quail, 1996). *Ubi1* was chosen for further manipulation of transgene

oil palm, particularly for driving constitutive expression of *bar* gene for selection of transformants on herbicide (Basta) (Masani and Parveez, 2008).

Most researches have used genes for resistance to antibiotics or herbicides, which may not be desirable for the final transgenic products. Kanamycin, geneticin (G418), neomycin and hygromycin antibiotics and the herbicide Basta™ were tested as selection agents to inhibit growth of untransformed cells. Hygromycin and Basta™ were found to be most suitable as selection agents for oil palm (Parveez et al., 1996, 2007). This success makes feasible any plan to manipulate oil palm by genetic engineering.

Table 2.4: Selectable marker genes used for the generation of stably transformed oil palm plants.

DNA delivery	**Selectable marker gene**	**Selection agent**	**References**
Biolistic	*bar* *hpt*	ppt hygromycin	Parveez et al., 1996; 2007
Agrobacterium	*bar*	ppt	Abdullah et al., 2005 Dayang Izawati et al., 2008
Biolistic	*gfp*	ppt	Na'imatulapidah and Parveez, 2007

2.5 Future prospects

Plant transformation technology offers an array of opportunities for basic scientific research and genetic modification of crops. The generation of transgenic plants requires the use of different selectable marker genes that are introduced together with the exogenous gene of interest. Recent progress in plant molecular biology and genome research has lead to a desire to introduce several genes into a single transgenic plant, necessitating the importance of various types of selectable marker genes. The most popular markers among the selectable marker genes applied to plant transformation are bacterial genes that confer resistance to the antibiotics (kanamycin and hygromycin) or to the herbicide (phosphinothricin). As these have been the target of concern among environmental authorities, scientists have been encouraged to develop alternative selection strategies (ACNFP, 1994), although such concerns may prove unfounded (Miki and McHugh, 2004). Development of environmentally-friendly marker-assisted selection system involving natural plant materials is gaining momentum. Utilization of selectable markers in crop plants fulfils the requirement for alternative selection markers in the development of genetically modified organisms. Earlier studies have supported the safe use of marker gene systems in plant transformation (Ramessar et al., 2007). In summary, selectable marker genes play a vital role to stimulate regeneration of transformed plant cells, thus supporting transgenic research of future plant biotechnologists and geneticists. Plant transformation works in monocotyledonous and dicotyledonous crops have proven that a wide range of selectable marker genes can be employed for successful genetic transformations. Selection systems do not pose any threat to mankind as evidenced by several experimental reports, thus leading to accelerated research towards the identification of new and novel marker gene systems from different sources.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals, enzymes and kits

All chemicals used in this study, unless otherwise stated, were obtained from Sigma Chemical Co., USA.

Restriction enzymes were obtained from New England Biolabs, Inc., Bacto-Trypton, Bacto-Agar and Bacto-Yeast Extract was purchased from Difco Laboratories, USA.

QIAprep Miniprep Kit, QIAquick Gel Extarction Kit, PCR Purification Kit and RNase free DNase Set were obtained from QIAGEN^{TM}.

MS (Murashige and Skoog, 1962) basal salts including vitamin, mannose, myo-innositol, plant growth regulators, plant agar were obtained from Sigma (St Louis, USA).

Ethanol, isoamylalcohol, methanol, galacial acetic acid, isopropanol and chloroform were obtained from UNILAB limited.

3.2 Plant materials

3.2.1 Oil palm

Oil palm embryogenic callus (EC) cultures were derived from *Elaeis guineensis* var. Tenera liquid suspension culture initiated from palm cabbage. This was kindly provided by Dr. Ahmad Tarmizi from Tissue Culture Group, MPOB.

3.2.2 Tobacco

Nicotiana tabacum L. used as a model plant was initiated from leaves of 30 –35 days old seedlings. They were cut into small pieces (2–4 mm) using razor blade and five leaf discs were cultured for each plate.

3.3 Determination of optimal concentration of mannose as a selection agent

Embryogenic calli (EC) of oil palm and leaf disc of tobacco were transferred onto selection medium supplemented with various combinations of mannose:sucrose – 0:30, 5:25, 10:20, 15:15, 20:10, 25:5 and 30:0 g/l. Five replicates were used for each treatment. In each plate, 0.5 g EC and 5 leaf discs were placed onto the solidified media and incubated in the dark at 28^{o}C and 25^{o}C, respectively. After 4 weeks, they were transferred onto fresh plates containing the same concentrations of mannose and sucrose under 16/8 h light conditions. The optimum concentration of mannose required for efficient selection against control tissues was determined by visual inspection and tissue weighing before subculture on fresh medium. The percentages for each treatment were calculated after 5 months of selection, the proliferation percentage (%) is calculated using the formula given by Parveez et al., 1996 as follows: (A – B) / (C – D) where A and B are the final and initial weights of the tissue/calli in the treated medium, respectively, C and D are the final and initial weights of tissues/calli in control medium, respectively. The proliferation percentage of the calli was used as a measure of mannose toxicity. The growth rate of the untreated control embryogenic calli exposed to 0:30 g/l

mannose:sucrose was used as a standard. The control was set to be at 100% proliferation. The means of five replicates were used in the final calculations.

3.3.1 Statistical analysis

For analysis (ANOVA) and Duncan's multiple range test (DMRT), the procedure of SPSS 8.0 version was used and calculated at the significance level of p• 0.05.

3.4 Plant transformation

3.4.1 Constructions of transformation vectors

The plasmids used for the construction of transformation vectors are listed in Table 3.1.

Table 3.1: Plasmids used in this study.

Plasmids	Description	Reference or Source
pBI221	Contain *gusA* gene driven by the CaMV*35S* promoter	Clontech
pAHC27	Contain *gusA* gene driven by the ubiquitin (*Ubi1*) promoter	USDA
pMB3	Contain CaMV*35S* promoter and *Nos* sequences	Masani et al. (2001)
pMB11	Contain ubiquitin (*Ubi1*) promoter and *Nos* sequences	Masani et al. (2001)

Table 3.2: Primer sequence for specific gene fragments.

Primer	Sequence
PMI-F	5'-GCG-CTA-GCC-ATG-GAA-AAA-CTC-ATT-AAC-TCA-G-3'
PMI-R	5'-GCG-CTA-GCT-TAC-AGC-TTG-TTG-TAA-ACA-CG-3'
NOS-R	5'-GGA-CTA-GTG-CTA-GCG-ATC-TAG-TAA-CAT-AGA-TGA-3'

3.4.1.1 Construction of pMI3 and pMI11

The phosphomannose isomerase (*pmi*) gene of *Escherichia coli* strains XL-1 Blue was cloned into PCR2.1TOPO vector by polymerase chain reaction (PCR) using the specific primers designed (Table 3.2). The resulted construct was verified by DNA sequencing and designed as PCRIIM2-68. The nucleotide sequences of a partial PMI mRNA from *Escherichia coli* as submitted to GenBank as included in Appendix A. The PCRIIM2-68 plasmid was digested with *Nhe*I to yield the 1.1 kb fragment, gel purified and ligated to the *Avr*II site of pMB3 and pMB11. The ligation mixture was transformed into DH5α competent cells and plated onto LB agar containing 50 µg/ml ampicilin and then incubated overnight at 37^{o}C. The 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 (Table 3.2). The two transformation vectors designated as pMI3 and pMI11 created were confirmed by digestion with *Bam*HI, *Bgl*II, *Eco*R1 and *Hind*III to check for the DNA fragments of expected size. The plasmid pMI3 contains the *pmi* gene under the control of constitutive promoter, CaMV*35S.* Plasmid pMI11 carries *pmi* gene driven by the maize ubiquitin (*Ubi1*) promoter.

3.4.1.2 Construction of pMI3G and pMI11G

The two vectors, pMI3 and pMI11, were modified by the introduction of *gusA* gene driven by the CaMV35S and *Ubi1* promoter, respectively. The DNA fragment of CaMV*35S-gusA-Nos* was excised from pBI221 by digestion with *Hind*III and *Eco*R1, gel purified and ligated to the *Hind*III and *Eco*R1 sites of pMI3 to create pMI3G. The ligation mixture was transformed into DH5α competent cells and plated on LB agar containing 50 µg/ml ampicilin and then incubated overnight at 37^{o}C. The positive clones were selected by *Hind*III and *Eco*R1 digestion. On other hand, the *Ubi1-gusA-Nos* fragment was excised from pAHC27 by *Hind*III digestion, gel purified and ligated

to the *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 ampicilin and then incubated overnight at 37^{o}C. The positive clones were selected by *Hind*III digestion.

3.5 Plasmid isolation using the Qiagen kit

The colonies with the bacteria containing the plasmids of interest were incubated overnight under shaking at 37^{o}C in either 10 ml or 200 ml LB medium, containing the appropriate antibiotic. The plasmids were isolated according to the kit manuals (Qiagen).

3.6 Preparation of glycerol stock

A total of 500 µl overnight bacteria culture and 500 µl of 50% glycerol was pipetted sequentially into a nunc tube, vortexed and cold frozen in liquid nitrogen. All activities were carried out aseptically in a laminar flow. The bacterial glycerol stock was stored at -70^{o}C.

3.7 Small scale plasmid isolation

Plasmid DNA from bacterial cultures was isolated using Mini s-prep Plasmid DNA extraction Kit GPM3001E (GeneTACG). Three ml of overnight grown culture were spun at 14000 rpm (Universal 32 R Hettich Zentrifugen, Germany) for 1 min and the medium was removed. The bacterial pellet was resuspended in 100 µl of G1 solution and subsequently incubated on ice for 2 min. Then, 200 µl of H2 solution was added and the mixtures were mixed gently at room temperature. The mixtures were incubated at room temperature for 1 min. K3 solution (350 µl) was added and the mixtures were incubated at room temperature for 1 min. The mixtures were centrifuged at 13,000 rpm

for 5 min in Micro 120 tabletop centrifuge (Hettich Centrifugen). The supernatant was transferred into the s-prep column and centrifuged at 10,000 rpm for 30 sec. The column was rinsed with 500 µl of wash solution and centrifuged at 10,000 rpm for 30 sec. The supernatant was discarded and the column was centrifuged again at 10,000 rpm for 30 sec to remove any residual solution. The column was transferred into a clean 1.5 ml microcentrifuge tube. Finally, DNA was eluted in 50 µl elution buffer.

3.8 Large scale plasmid isolation

Large scale plasmid isolation was carried out using QIAGEN^{TM} Plasmid Purification kit. A single colony from freshly streaked selective plate was picked and inoculated in 5 ml LB medium containing appropriate selective antibiotic. The starter cultures were incubated for approximately 8 h at 37^{o}C with vigorous shaking. The cultures were diluted 1/5000 to 1/1000 (100 • l - 200 • l of starter cultures were inoculated in 100 ml medium). The cultures were incubated at 37^{o}C for 12-16 h with vigorous shaking. Bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4^{o}C. Bacterial pellet was resuspended in 10 ml Buffer P1. Buffer P2 (10 ml) was added and mixed thoroughly by vigorously inverting the sealed tube for 4-6 times. The suspensions were incubated at room temperature for 5 min. The mixtures were added with 10 ml buffer P3, mixed immediately and thoroughly by vigorously inverting for 4-6 times. The mixtures were incubated on ice for 20 min and subsequently centrifuged at • 20 000 rpm for 30 min at 4^{o}C. The supernatant containing plasmid DNA was removed promptly. QIAGEN tip 500 was equilibrated by applying 10 ml Buffer QBT and allowed the column to empty by gravity flow. The supernatant containing plasmid DNA was applied to the QIAGEN tip. The QIAGEN tip was washed with 2 x 30 ml buffer QC and the DNA was eluted with 15 ml buffer QF. DNA was precipitated by adding 10.5 ml (0.7 volumes) room temperature isopropanol to the eluted DNA. The mixtures were

mixed and centrifuged immediately at 15,000 x g for 30 min at 4^{o}C. The DNA pellet was washed with 5 ml 70% ethanol and centrifuged at 15,000 x g for 10 min. The DNA pellet was subsequently air dried and redissolved the DNA in a suitable volume of TE buffer.

3.9 Restriction enzyme digestion

All of the restriction enzyme digestions were carried out using the conditions recommended by the supplier of the specific enzyme. Restriction enzyme digestions were usually performed for screening of recombinant clones, digesting plant genomic DNA and isolating specific DNA fragments for subcloning and modification. Typically, the digestions were carried out in a 20 µl reaction containing 1 unit enzyme and 1 µg of DNA. One unit enzyme is defined as the amount of the enzyme required to completely digest 1 µg of DNA at the recommended temperature for 1 h. For digestions using two restriction enzymes, the digestions were performed simultaneously if the reaction conditions were optimum for both of the enzymes. Otherwise, the reactions were performed sequentially with an intermediate phenol extraction step to remove the first enzyme and buffer.

3.10 Agarose gel electrophoresis

Agarose gel electrophoresis can be used for separating, identifying and purifying 0.5 to 25 kb DNA fragments (Sambrook et al., 1989). The fragments of DNA were separated by electrophoresis through an agarose gel (0.7-4%), depending on the size of fragments. The agarose gel was prepared by heating the agarose in 1XTAE buffer (10 mM Tris; 5 mM sodium acetate; 0.5 mM EDTA, pH 8) in a microwave oven until the agarose is completely dissolved. The gel solution was allowed to cool and poured into gel trays. The DNA samples to be loaded were mixed with loading buffer (0.25% Xylencyanol;

0.25% Bromophenol blue; 50% Glycerol; 0.5 M EDTA, pH 8) and electrophoresed in 1X TAE containing 0.5 µg/ml ethidium bromide (EtBr). Electrophoresis was carried out at 100 volts for 90 min. The gel was viewed under ultraviolet (UV) transilluminator and images were processed and recorded using Imager Kit Digital Alpha 2200 (Kodak).

3.11 Isolation of DNA fragments from agarose gel

The DNA that was required for subcloning procedures was isolated by the means of agarose gel electrophoresis. The desired DNA fragments were excised using a clean razor blade and isolated using the gel extraction kit (QIAGEN).

3.12 DNA purification by gel extraction

Fragments generated through PCR amplification were excised from gel by scalpel and placed in the 1.5 ml microcentrifuge tube. The fragments were purified from the gel slabs using QIAEXII kit (QIAGEN). Three volume of QX1 buffer and 10 µl of QIAEXII solution were added to the fragments. The mixtures were incubated at 50°C for 10 min to dissolve the gel slab. To ensure the gel slab has completely dissolved, mixtures were mixed by inverting every 2 min during the incubation. The samples were centrifuged for 30 sec and the supernatant was removed. The pellet was washed with 500 •l of QXI buffer followed by centrifugation at 13000 rpm for 30 sec. The supernatant was removed and the resin was resuspended in 500 •l of PE buffer. Then, the sample was centrifuged for 30 sec and the supernatant was removed. This washing step was repeated for three times. Finally, pellet was air-dried and the DNA was eluted in 15 •l of sterile water.

3.13 Dephosphorylation

Self-ligation of vector DNA could lead to a reduction in the frequency of recombinant plasmids. This can be prevented by removing the 5' phosphate group from the vector DNA. The dephosphorylation step was performed in 60 µl reaction mixtures containing 10 units of Calf Intestinal Phosphatase (CIP) and 1X reaction buffer (Promega). A ten-fold concentrated reaction buffer (0.5 M Tris-HC1, pH 9; 10 mM $MgCl_2$; 1 mM $ZnCl_2$; 10 mM Spermidine) was supplied by Promega. The mixtures were incubated for 1 h at 37°C and the reaction was terminated by heat-inactivation at 65°C for 15 min**.** The reaction mixtures were extracted by phenol extraction, ethanol precipitation and the DNA was resuspended in 20 µl of sterile water.

3.14 Ligation

The ligations were performed in a 20 •l reaction mixtures containing 1X ligation buffer at 16°C overnight. The DNA insert used was either equal or up to 3 fold molar concentration over DNA vector. For ligation of DNA with sticky ends, only 1 unit of T4 DNA ligase (Research Biolabs) was used. For the DNA with blunt ends, the reaction was supplemented with 2 •l of 40% PEG 4000 and 2 units of T4 DNA ligase. The resulting reaction mixtures were incubated at 65°C for 10 min to inactivate T4 DNA ligase.

3.15 Preparation of *Escherichia coli* competent cells

The preparation of *E. coli* competent cells was performed according to the method of Hanahan (1982). All centrifugations were performed at 4°C. A 250 ml flask containing 100 ml LB broth was inoculated with 1 ml of an overnight *E. coli* culture. The cells were grown at 37°C with constant shaking at 160 rpm. When the optical density (600 nm) of the culture was between 0.3 and 0.4, cells were harvested by centrifugation at

9,000 rpm for 10 min. Cells were subsequently resuspended in 10 ml Tfb1 buffer (100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% glycerol, pH 5.8), and incubated on ice for 5 min. The cells were again harvested at 9,000 rpm for 5 min and resuspended in 1 ml ice cold TfbII buffer (10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% glycerol, pH 6.5), and incubated on ice for 15 min. The cells were pipetted into 100 µl aliquots, frozen immediately in liquid nitrogen and stored at -80°C until required.

3.16 Transformation of *Escherichia coli* competent cells

The entire volume of a ligation reaction or 5 µl (~10 ng) of plasmid DNA was gently mixed with a 100 µl aliquot of *E. coli* cells thawed on ice. The mixture was incubated on ice for 1 h and subjected to a 90 sec heat shock at 42°C followed by a cold shock on ice for 2 min. The cells were incubated at 37°C for 1 h followed by the addition of 900 µl LB broth pre-warmed to 37°C. The cells were spread on LB agar (0.5% Yeast extract, 1% Tryptone, 1% NaCI, 0.75% agar, pH 7) containing the appropriate antibiotic and incubated overnight at 37°C.

3.17 Polymerase chain reaction (PCR) amplification

PCR amplification was performed using MJ Research Programmable Thermal Controller (PTC-100^{TM}). Amplifications were carried out in a 25 •l final volume. Between 10 ng plasmid DNA or 100 ng genomic DNA were used as template, depending on experiments. Each PCR reaction 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 PMI-F and NOS-R primers as described in the Table 3.2, 0.2 µl 5U/µl *Taq* polymerase (Promega) and appropriate amount of template DNA. Total volume is made up with distilled water. The PCR reaction condition is shown in the table 3.3. Amplification products were separated in a 1% (w/v) agarose gel. The PCR products

were purified by using QIAquick PCR Purification Kit (QIAGEN) for uses such as cloning.

Table 3.3: PCR conditions for amplification of specific gene fragments.

	Temperature (^{o}C)	**Time (min)**		**Cycles**
Denaturation	95	5	----	1
Denaturation	95	1	}	30
Annealing	60	1		
Elongation	72	3		
Elongation	72	5	----	1

3.18 Purification of PCR products

PCR products required for restriction enzyme digestions and subsequent cloning were purified using the QIAquick PCR Purification Kit (QIAGEN). For every 100 µl PCR product, 500 µl PB buffer were added and vortexed to mix the solution. A QIAquick spin column was placed into a 2 ml collection tube and the sample was loaded into it. The column was centrifuged for 1 min and washed with 0.75 ml PE buffer and centrifuged for 30 sec to remove any residual buffer. The column was placed in a new centrifuge tube. To elute the DNA, 50 µl EB buffer was added to the column and centrifuged for 30 sec.

3.19 Biolistic transformation

All four vectors carrying *pmi* gene were used for transformation. Plasmid DNA was extracted using the Qiagen Maxi Preparation Kit. The DNA was precipitated and resuspend in 1 ml TE buffer to a final concentration of 300-500 μg/μl.

3.19.1 Preparation of target material for transformation

Oil palm EC and tobacco leaf discs were cultured on agar solidified medium containing embryoid and callus initiation medium, respectively (Table 3.4). All tissues were arranged in the center of a petri dish (approximately 3 cm in diameter) and incubated in the dark at 28^{o}C, 24 h before bombardment.

Table 3.4: Medium composition for embryoid development of oil palm and tobacco.

	Oil palm medium	***Tobacco medium***
MS salts including Vitamin (g/l)	4.4	4.4
BAP (mg/ml)	-	1.0
Sucrose (g/l)	30.0	30.0
Plant agar (g/l)	8.0	8.0
pH	5.8	5.8

3.19.2 Preparation of gold particle

Microcarrier preparation was carried out according to the Biolistic PDS-1000/He Particle Delivery System Instruction Manual (BioRad). The gold particles were prepared by placing 60 mg of gold particles in 1 ml of 100% ethanol and vortexed for 3 min. The gold particles were centrifuged at 10,000 rpm for 10 sec followed by the removal of supernatant. The gold particles were washed with 1 ml sterile water, vortexed and centrifuged before supernatant was removed. The procedure was repeated.

The gold particles were resuspended in 1 ml sterile water and dispensed into 100 µl aliquots and stored at 4°C.

3.19.3 Bombardment of plant tissues

To each aliquot of 100 µl gold particles, 20 µg DNA, 100 µl 2.5 M $CaCl_2$, and 40 µl 0.1 M spermidine were added in order, with continuous vortexing. The vortexing was continued for 3 min followed by centrifugation at 10,000 rpm for 10 sec. The supernatant was removed as much as possible and the particles were washed twice with 500 µl 100% ethanol, followed by mixing, and centrifugation at 10,000 rpm for 60 sec. Finally, the DNA coated gold particles were resuspended in 120 µl absolute ethanol. For each bombardment, 6 µl of DNA coated gold particles was loaded onto the centre of the macrocarrier and dried under a sterile condition. Bombardment was carried out 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 mm Hg vacuum pressure (Parveez, 1998). After the gene delivery, tissues were incubated in the dark at 28^{o}C for callus initiation.

3.19.4 Selection and regeneration

One month after gene delivery, bombarded oil palm EC and tobacco leaf disc were moved for selection on MS medium modified to contain 30 g/l mannose as the selection agent. Sucrose was not added to the selection medium; mannose served as the only carbohydrate source in cultures. This was followed by growth in the 16/8h light conditions at 28^{o}C and 25^{o}C, respectively. The medium was changed every four weeks. After a period of 3-6 months, the regenerated oil palm shoots were placed on shoot inducing medium and finally in the rooting medium (Table 3.5). For tobacco explants, to induce shoots elongation and rooting, the callus obtained were cultured onto MS0

medium (Table 3.5) containing 30 g/l mannose instead of sucrose. Approximately 3-4 months from bombardment to produce transgenic plantlet.

Table 3.5: Selection medium for *in vitro* culture of *E. guineensis.*

	EC medium	***SIM medium***	***Rooting medium***
MS salts including Vitamin (g/l)	4.4	4.4	4.4
MyoInositol (g/l)	0.1	0.1	0.1
NAA (μM)	0.1	0.1	9.0
L-Glutamine (g/l)	0.1	0.1	0.1
L-Arginine (g/l)	0.1	0.1	-
L-Asparagine (g/l)	0.1	0.1	-
Activated charcoal (%)	-	-	0.15
Plant agar (g/l)	8.0	8.0	8.0
Mannose (g/l)	30.0	30.0	30.0
Plant agar (g/l)	8.0	8.0	8.0
pH	5.8	5.8	5.8

3.20 Analysis of putative transformants

3.20.1 Histochemical GUS assay

Expression of the GUS gene was assayed using a modified protocol of Jefferson (1989). Two vectors carrying the GUS reporter gene driven by the CaMV*35S* (pMI3G) and the *Ubi1* promoter (pMI11G) were bombarded into oil palm embryogenic calli and tobacco leaf disc. The experiment was carried out in 5 replicates. The bombarded tissues were incubated in the dark at 28^{o}C, 48 h. The tissues were later stained overnight (20 h) at 37^{o}C in GUS buffer (0.2 M sodium phosphate buffer (pH 7.0), 0.5 mM $K_4[Fe(CN)_6]$; 0.5 mM $K_3Fe(CN)_6$), X-GlucA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; 0.5 mg/ml) dissolved in DMSO, was added to the buffer. Blue spots were scored optically using a Nikon UFX-DX system.

3.20.2 Total DNA isolation

Total DNA extraction from oil palm embryoids, plantlet and tobacco plantlet was carried out using modified Doyle and Doyle (1987) method. Three g of samples were ground to very fine powder in liquid nitrogen using mortar and pestle. The powder was then transferred into SS34 tubes. The samples were homogenized with 10 ml CTAB buffer (100 mM Tris-HCl ph 8, 20 mM EDTA pH 8, 1.4 M NaCl, 2% CTAB, and 0.2% • -mercaptoethanol) and incubated at 65ºC for 1 h. Five ml chloroform:isoamyl alcohol (24:1) were added and the mixture were centrifuged at 4ºC, 13,000 rpm for 15 min. The clear upper layer (aqueous phase) was transferred into a new tube and 5 ml chloroform:isoamyl alcohol (24:1) were added. The mixture was then centrifuged at 4ºC, 13,000 rpm for 15 min. The aqueous phase was transferred to a SS34 tube and 0.6 volume of cold isopropanol was added. The mixture was mixed gently and incubated at -20^{o}C overnight. On the following day, DNA was collected by centrifuging at 4^{o}C, 13,000 rpm for 15 min. The supernatant was discarded and the pellet was washed with 10 ml washing buffer (76% ethanol, 1 mM ammonium acetate). The buffer was discarded and the pellet was air-dried and dissolved in 500 µl TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). Then, 2 µl RNAse was added into the samples and incubated at 37ºC for 15 min. The purity and concentration of DNA was determined using spectrophotometer. DNA samples were stored at 4^{o}C until used.

3.20.3 Total RNA isolation

Total RNA extraction from oil palm tissues including embryoids and plantlet was carried out according to modified Doyle and Doyle (1987) method. Three g of samples was frozen in liquid nitrogen and ground to fine powder with mortar and pestle. Then, the powdered tissue was homogenized in 15 ml extraction buffer [2% CTAB, 2% polivinylpyrrolidone (PVP-40), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2

M NaCl, 0.05% spermidine trihydrochloride, 2% β-mercaptoethanol (added just before use)] previously pre-warmed to 55^{o}C. The homogenate was incubated at 55^{o}C for 15 min with vigorous shaking for several times. Equal volume of chloroform: isoamylalcohol (24:1) was added and the phases were separated by centrifugation at 6500 rpm for 15 min at 4^{o}C. The aqueous layer was removed to a new centrifuge tube and extracted once with equal volume of chloroform:isoamylalcohol (24:1) by centrifugation at 6500 rpm (4^{o}C) for 15 min to pellet and discard insoluble materials. The aqueous layer was then transferred to a new tube without disturbing the chloroform drop. 8 M LiCl was added to final concentration of 2 M. The mixture was mixed by inversion and incubated overnight at 4^{o}C to precipitate the RNA. The RNA was pelleted by centrifugation at 13,000 rpm (4^{o}C) for 20 min. The RNA was dissolved in 400 µl 3M sodium acetate and precipitated using 100% ethanol by centrifugation at 13,000 rpm for 20 min, rinsed with 70% cold ethanol and air-dried. The RNA was dissolved in RNase-free water and stored at –80^{o}C until used. The electrophoresis was performed in 1% agarose gel and 1x TAE buffer (0.04 M Tris-Cl pH7.2, 0.02 M sodium acetate, 0.001 M EDTA). A sample with a good integrity is indicated by the presence of two ribosomal RNA bands (28S and 18S).

3.20.4 Removal of DNA contaminant

Total RNA from embryoids and plantlets was isolated using protocol described in Section 3.20.3. Removal of DNA contaminant from the total RNA samples was carried out using the Qiagen RNase free DNase Set (QIAGEN). A total of 10 µg of total RNA was diluted to a final volume of 100 µl using RNase free water and mixed with 350 µl RLT buffer and 250 µl 100% ethanol. The mixture was applied to an RNeasy mini column placed in a 1.5 ml collection tube. Then the column was centrifuged at 12,000 rpm in Micro 120 tabletop centrifuge (Hettich Centrifugen) for 15 sec. RW1 buffer (350

µl) was added to the column and the centrifugation was repeated. DNase I (30 units, diluted in RDD buffer) was then pippetted directly onto the silica gel membrane. The column was left at room temperature for 15 min. Then, 350 µl of RW1 buffer was added to the column followed by centrifugation at 12, 000 rpm for 15 sec. The column was washed twice with 500 µl RPE buffer. Finally, the RNA was eluted in 10 µl RNase free water.

3.20.5 Determination of DNA and RNA concentrations by spectrophotometer

The concentrations of DNA and RNA were determined using NanoDrop ND-1000 Spectrophotometer (NanoDrop) at an extinction of λ=260 nm, by using the formula; DNA concentration (OD_{260}.D.50 µg/ml), RNA concentration (OD_{260}.D. 40 µg/ml), where D is dilution factor. The purity of DNA and RNA samples were determined by ratio between the OD_{260}/OD_{280}. A pure sample has a ratio of 1.8 and 2.0 for DNA and RNA, respectively.

3.20.6 Polymerase chain reaction (PCR) amplification

PCR analysis has been carried out to prove successful gene transfer. Approximately 600-2000 ng of sample DNA was used for each reaction, while 100 ng of plasmid DNA was used as positive controls. Each PCR reaction was carried out in 25 µl reaction mixture 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 and 0.2 µl 5U/µl Taq polymerase (Promega), and appropriate amount of template DNA. Total volume is made up with distilled water. The primers used are shown in the Table 3.2. PCR was carried out using PTC-100^{TM} Programmable Thermal Controller (MJ

Research, Inc.). A 1.1 kb band size is expected to be amplified using PMI-F and PMI-R primers. The PCR products were electrophoresed on a 1.2 % agarose gel at 110V for 80 min. The PCR reaction condition for the PMI primers for the *pmi* gene is shown in the Table 3.6.

Table 3.6: PCR and RT-PCR conditions for amplification of fragments of the *pmi* gene.

	Temperature (^{o}C)	**Time (min)**		**Cycles**
Denaturation	94	5	------	1
Denaturation	94	1	}	
Annealing	50	1	}	34
Elongation	72	1 and 05 sec/cycle	}	
Elongation	72	5	------	1

3.20.7 Reverse transcriptase

Intact RNA was converted cDNA by using High Capacity cDNA Achive Kit (Applied Biosystem). The conversion reaction was performed using 10 µg of the treated total RNAs. Reaction mixtures (final volume of 50 µl) containing 10 µg of RNA, 1X reverse transcriptase buffer, 1X dNTPS, 1X random primers, 0.6 µl RNase inhibitor and 125 U of reverse transcriptase were assembled in 0.5 ml tubes. The reaction mixtures were incubated at 25 °C for 10 min and followed by incubation at 37 °C for 120 min. The mixtures were kept at -20 °C until used.

3.20.8 Reverse transcription (RT)-PCR

For semi quantitative RT-PCR, total RNA treated with DNAse to prevent amplification of fragments from genome. The cDNA was directly used for PCR reaction. The reactions were performed in tubes containing 5 µl 10X RT Buffer, 2 µl 25X dNTPs, 5 •l of gene specific primer (PMI-F and PMI-R, 18S-R and 18S-F), 1.25 µl RNA

inhibitor, 2.5 µl of RT (ABI, Inc), 2 •g of first strand cDNA and RNase free water. Complete reaction mixes were aliquoted and placed into a PTC-100^{TM} Programmable Thermal Controller (MJ Research, Inc.). The PCR reaction condition for the *pmi* gene is shown in the Table 3.6. PCR products were separated electrophoretically and visualized by ethidium bromide staining.

3.20.9 Sequencing and identification of PMI

The PCR products were confirmed by direct sequencing using automated DNA sequencer (ABI PRISM Model 377 Version 3.4) with 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 sequence alignment DNA. Protein homology search against the GenBank databases was performed using BLAST 2.0 (Altschul et al., 1997).

CHAPTER 4

RESULTS AND DISCUSSIONS

Transformation of oil palm embryogenic callus method has mainly been carried out either via biolistic or *Agrobacterium tumefaciens* method using an antibiotic or herbicide resistance genes as selectable marker gene. However, the use of alternative selectable marker genes is needed due to the public concern on the transgene, which should not present risks to human, animals and to the environment. As an alternative, system based on phosphomannose isomerase as the positive selectable marker gene and mannose as the selective agent was successfully applied here for oil palm transformation.

The present work involved 4 major activities. The first phase was to construct four vectors carrying the *pmi* gene. The second phase was on the determination of the optimal concentration of mannose as a selection agent for oil palm. In this experiment, a various concentrations of mannose were tested on oil palm embryogenic callus, ranging from 0.5 to 30 g/l. The third phase involved the transformation of the vectors into oil palm embryogenic callus using biolistic method. Finally, molecular analyses to determine gene integration as proof of stable transformation process.

4.1 Construction of PMI transformation vectors

Four vectors carrying the *pmi* gene were engineered to allow for transformation into oil palm using positive selection via biolistic method. The pMB3 and pMB11 vectors (Masani et al., 2001) carrying CaMV*35S* and *Ubi1* promoters, respectively, were used as backbone vectors for inserting *pmi* gene. All four vectors constructed for expressing *pmi* gene were designated as pMI3, pMI3G, pMI11 and pMI11G (Table 4.1).

The two constitutive promoters, CaMV*35S* and *Ubi1* promoters were chosen to drive *pmi* gene in this study because it was proven effective in oil palm transformation studies (Parveez, 1998). The CaMV*35S* promoter used in a number of different plant species have been shown to be constitutive and highly active especially in dicot plants, whereas, *Ubi1* promoter has been characterized as suitable promoter for monocot plants.

gusA gene as a reporter gene was introduced into pMI3G and pMI11G vectors. The reporter gene could be used to monitor transgenic events and manually separate transgenic material from non-transformed material. All vectors were sequenced to confirm the integrity of the T-DNA region. Based on the complete sequence of each vector, restriction map was illustrated by using the Vector NTI software (Invitrogen). The size of vectors, position of restriction sites and the size of generated fragments for all constructed vectors are shown in Appendices B-E.

Table 4.1: List of vectors constructed in this study.

Ligation	Vectors	Size	Description
PCRIIM268/*Nhe*1+ pMB3/*Avr*II	pMI3	5278 bp	*CaMV35S-pmi-Nos*
PCRIIM268/*Nhe*1+ pMB11/*Avr*II	pMI11	6401 bp	*Ubi1-pmi-Nos*
pMI3 + pBI221/*Hind*III/*Eco*RI	pMI3G	8221 bp	*CaMV35S-pmi-Nos-CaMV35S-gusA-Nos*
pMI11 + pAHC27/*Hind*III/*Eco*RI	pMI11G	10518 bp	*Ubi1-pmi-Nos-Ubi1-gusA-Nos*

4.1.1 Construction of pMI3 and pMI11

Initially, for the construction of pMI3 and pMI11 vectors as shown in Figure 4.1 and Figure 4.2, the *pmi* gene was isolated from *Escherichia coli* strains XL-1 Blue by polymerase chain reaction (PCR) to generate a 1193 bp fragment. The fragment was cloned into PCR2.1TOPO (3931 bp) to generate PCRIIM2-68 (5124 bp). PCRIIM2-68 was sequenced for verification. Plasmid was confirmed by digestion using *Nhe*I to yield the 1183 bp fragment (Figure 4.3). The 1183 bp fragment of *pmi* gene was later inserted into the *Avr*II site of pMB3 (4095 bp) and pMB11 (5218 bp), respectively. Expected clones were screened by *Bam*HI digestion to cleave the 2320 bp of CaMV*35S-pmi-Nos* and 3440 bp of *Ubi1-pmi-Nos* fragment as shown in Figure 4.4 and Figure 4.5, respectively. The inserted gene was confirmed to be in sense orientation to the CaMV*35S* and *Ubi1* promoter by PCR analysis using forward primer, PMI-F and reverse primer, NOS-R (Table 3.5). PCR products of approximately 1.4 kb for *pmi-Nos* were amplified as shown in Figure 4.6 and Figure 4.7. All plasmids generated (pMI3 and pMI11) were also confirmed by digestion with *Bam*HI, *Bgl*II, *Eco*R1 and *Hind*III to yield the expected fragments 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 4.8 and Figure 4.9.

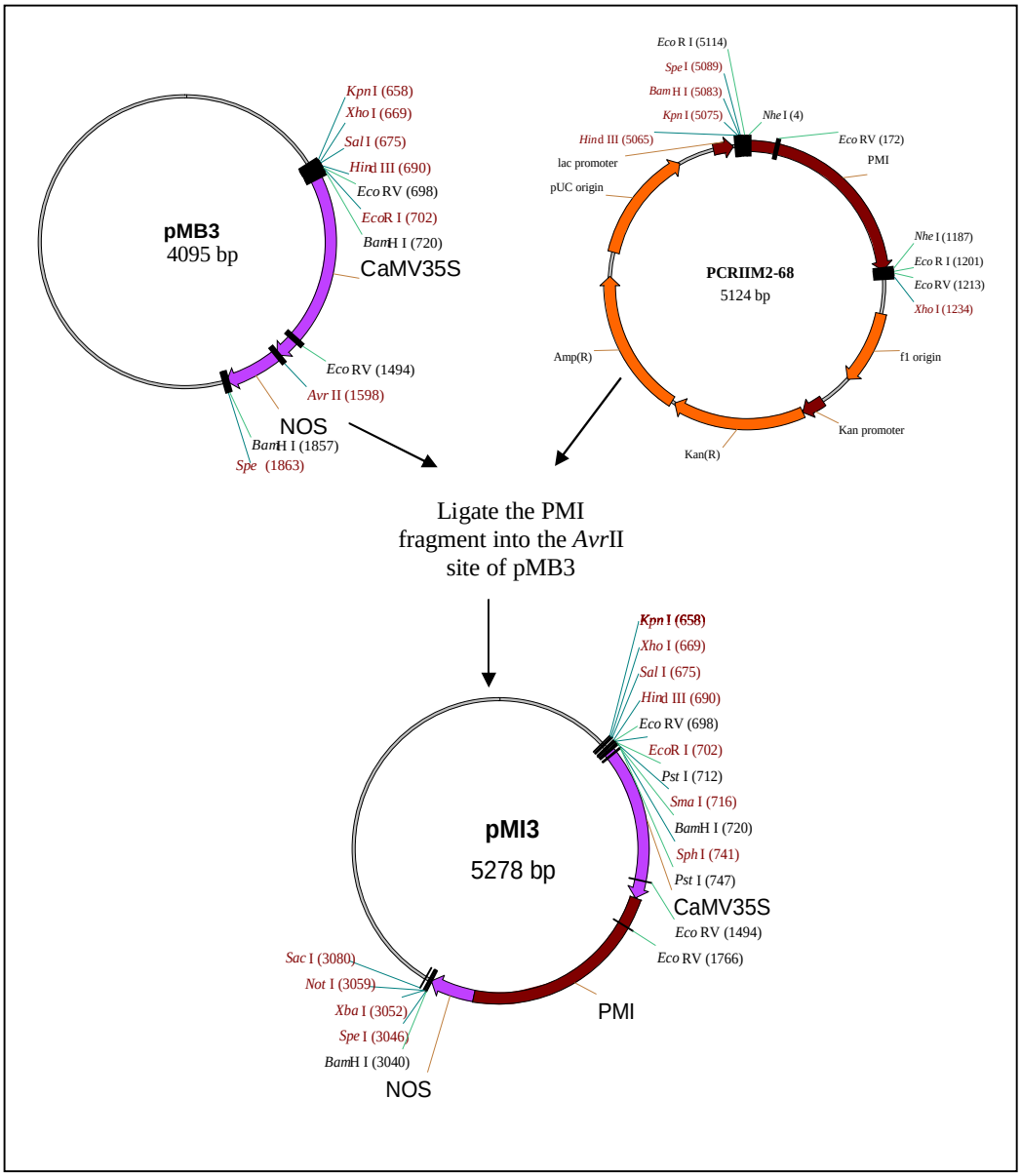

Figure 4.1: Construction of pMI3. The PMI fragment excised from the *Nhe*I digestion of plasmid PCRIIM2-68. Gel purified and ligated to the *Avr*II site to create pMI3.

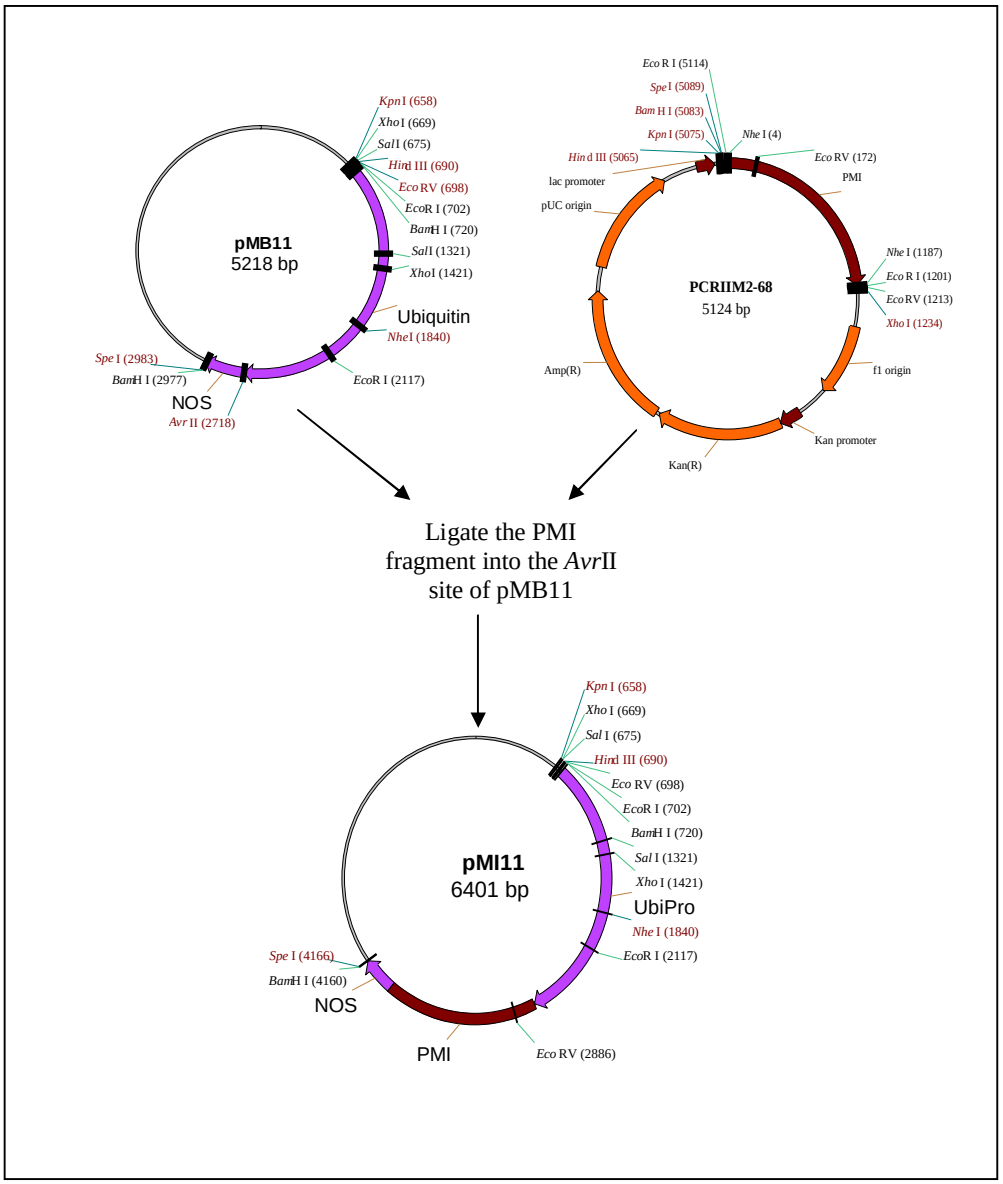

Figure 4.2: Construction of pMI11. The PMI fragment excised from the *Nhe*I digestion of plasmid PCRIIM2-68. Gel purified and ligated to the *Avr*II site to create pMI11.

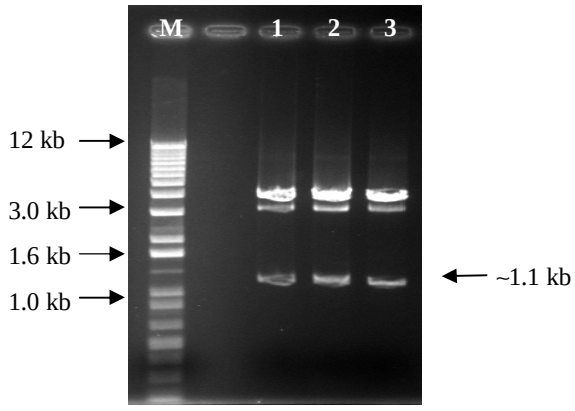

Figure 4.3: Purified DNA was digested with *Nhe*I. Lane M: 1 kb plus DNA ladder, lanes 1, 2 and 3: Positive clones for PCRIIM2-68. Arrow indicates the size of fragment, 1.1 kb.

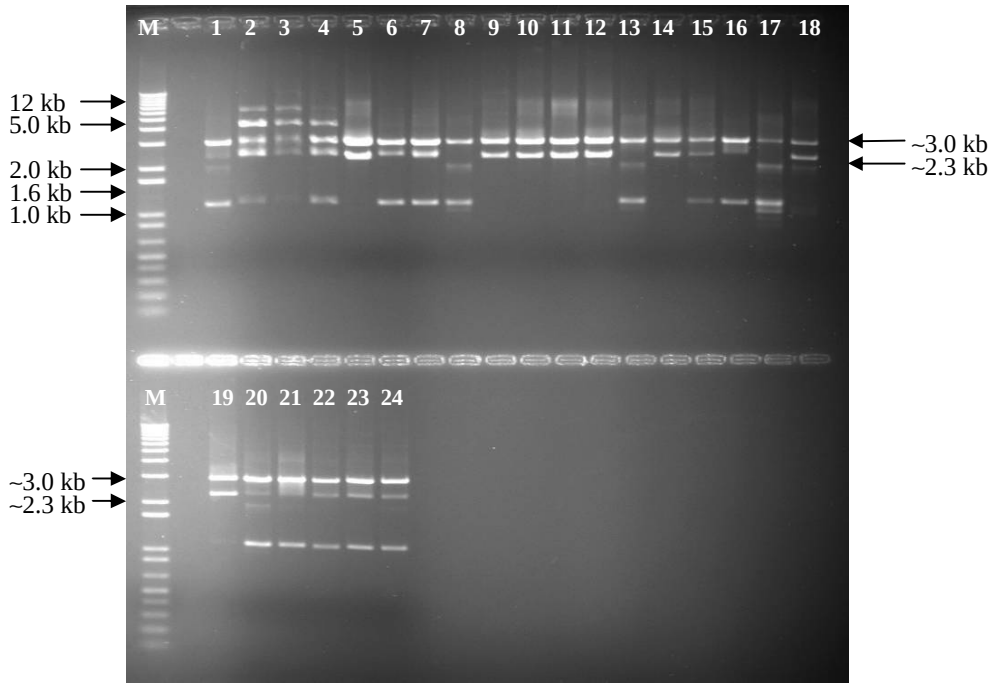

Figure 4.4: Selection of pMI3. Purified DNA was digested with *Bam*H1. Lane M: 1 kb plus DNA ladder; lanes 5, 9, 10, 11, 12, 14, 19: Positive clones for pMI3 vector. Arrow indicates the size of fragments, 2.3 kb of CaMV*35S-pmi-Nos* and 3.0 kb of pBlueskriptSK.

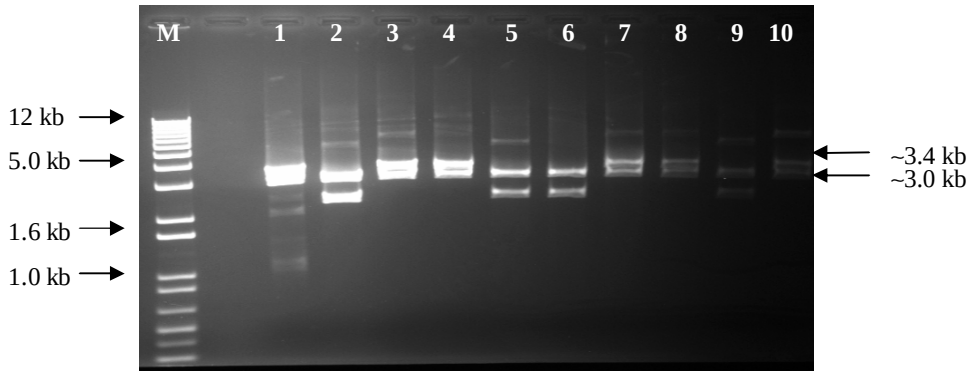

Figure 4.5: Selection of pMI11. Purified DNA was digested with *Bam*H1. Lane M: 1 kb plus DNA ladder, lanes 1, 3, 4, 7, 8 and 10: Positive clones for pMI11 vector. Arrow indicates the size of fragments, 3.0 kb of pBlueskriptSK and 3.4 kb of *Ubi1-pmi-Nos*.

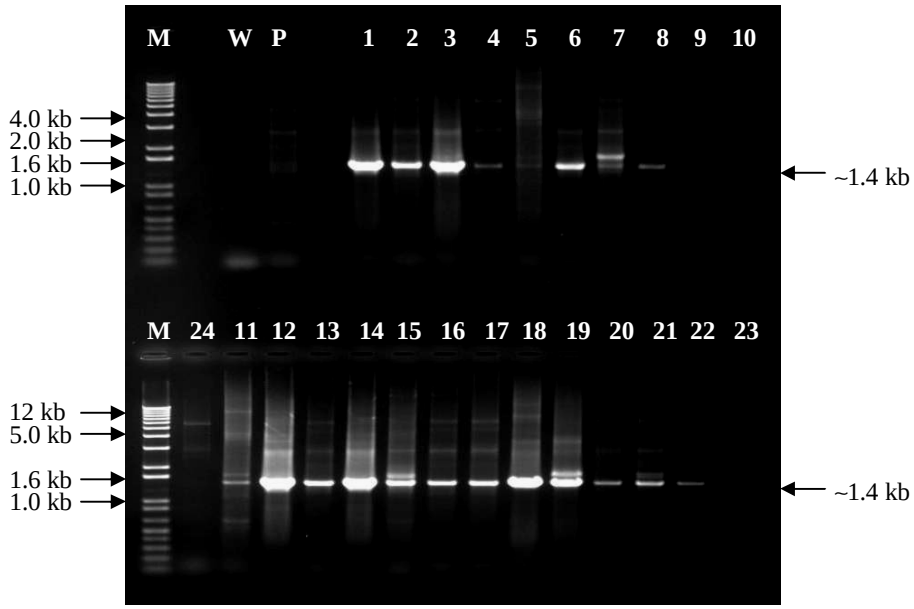

Figure 4.6: Confirmation of sense orientation of inserted gene in pMI3 by PCR analysis. Lane M: 1 kb plus DNA ladder; W: water control; P: pMB3 plasmid; lane 1-3, 6, 8, 11-22: sense *pmi* gene in pMI3. Arrow indicates the PCR products sized 1.4 kb.

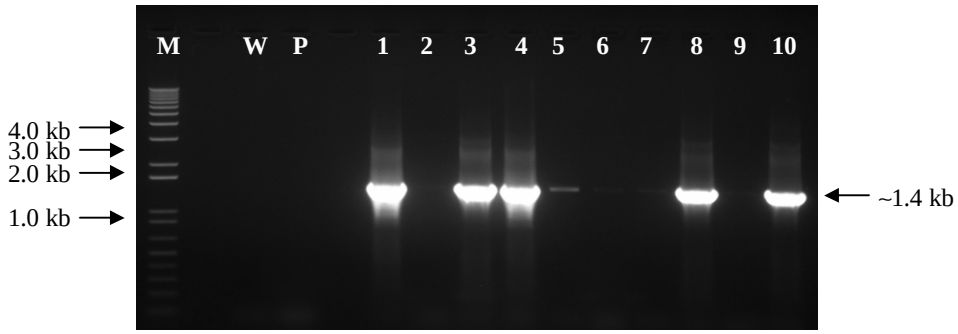

Figure 4.7: Confirmation of sense orientation *pmi* gene in pMI11 by PCR analysis. Lane M: 1 kb plus DNA ladder; W: water control; P: pMB11 plasmid; lane 1-10: positive clones of pMI11. Arrow indicates the PCR products sized 1.4 kb.

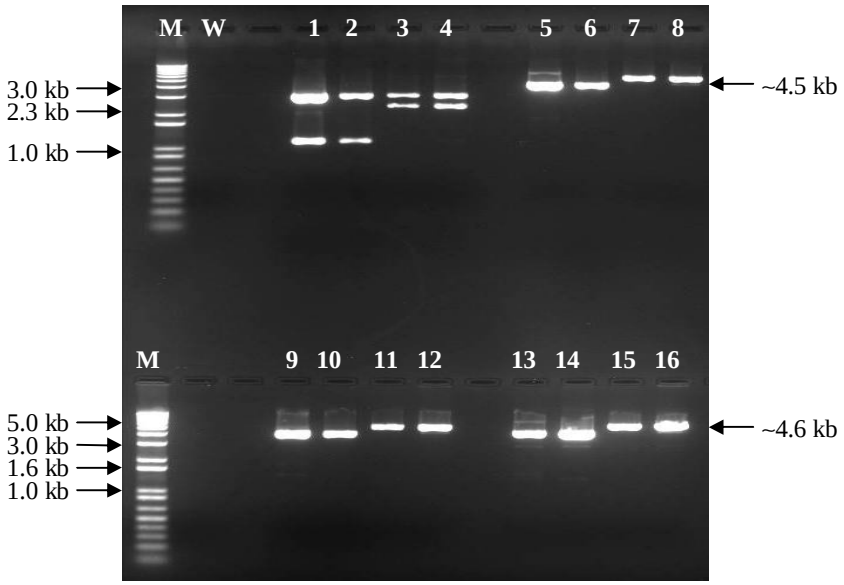

Figure 4.8: Restriction endonuclease analysis of pMB3 (lanes 1, 2, 5, 6, 9, 10, 13 and 14) and pMI3 (lanes 3, 4, 7, 8, 11, 12, 15 and 16). Equal amount of DNA of each plasmid was digested with different restriction endonucleases (lane 1-4: *Bam*HI; lane 5-8: *Bgl*II; lane 9-12: *Eco*RI; lane 13-16: *Hind* III). Lane M: 1 kb plus DNA ladder. The size of the observed fragments in each digestion was as predicted.

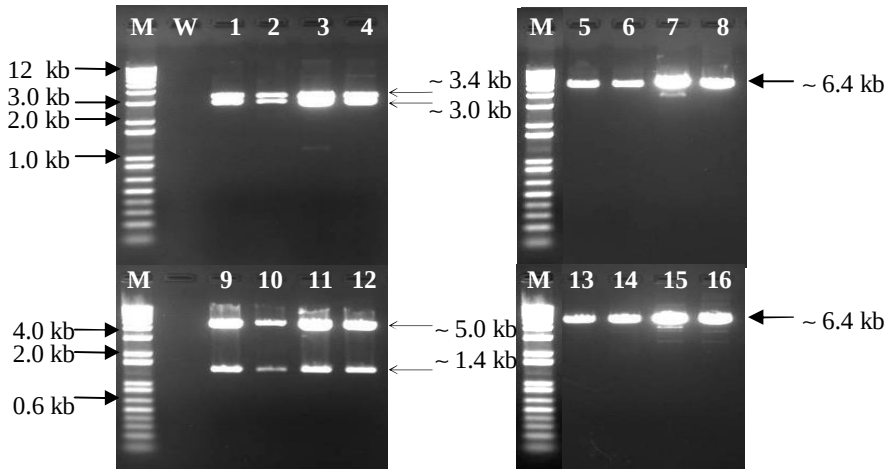

Figure 4.9: Restriction endonuclease analysis of pMI11. Equal amount of DNA of each plasmid was digested with different restriction endonucleases (lane 1-4: *Bam*HI; lane 5-8: *Bgl*II; lane 9-12: *Eco*RI; lane 13-16: *Hind*III). The size of the observed fragments in each digestion was as predicted. Lane M: 1 kb plus DNA ladder.

4.1.2 Construction of pMI3G and pMI11G

The pMI3G vector as shown in Figure 4.10 has been constructed by introducing the CaMV*35S-gusA-Nos* fragment from pBI221 (Figure 4.13) into the *Hind*III and *Eco*R1 site of pMI3 (Figure 4.12). Selected clones were screened by *Hind*III and *Eco*R1 digestion to cleave the 8.2 kb of CaMV*35S-pmi-Nos*-CaMV*35S-gusA-Nos* fragment as shown in Figure 4.15. Similar approach was used to generate pMI11G (10.5 kb) vector shown in Figure 4.11. *Ubi1-gusA-Nos* fragment from pAHC27 (Figure 4.14) was introduced into the *Hind*III site in pMI11 (Figure 4.12). The size of the insert was confirmed by digestion with *Hind*III as shown in Figure 4.16.

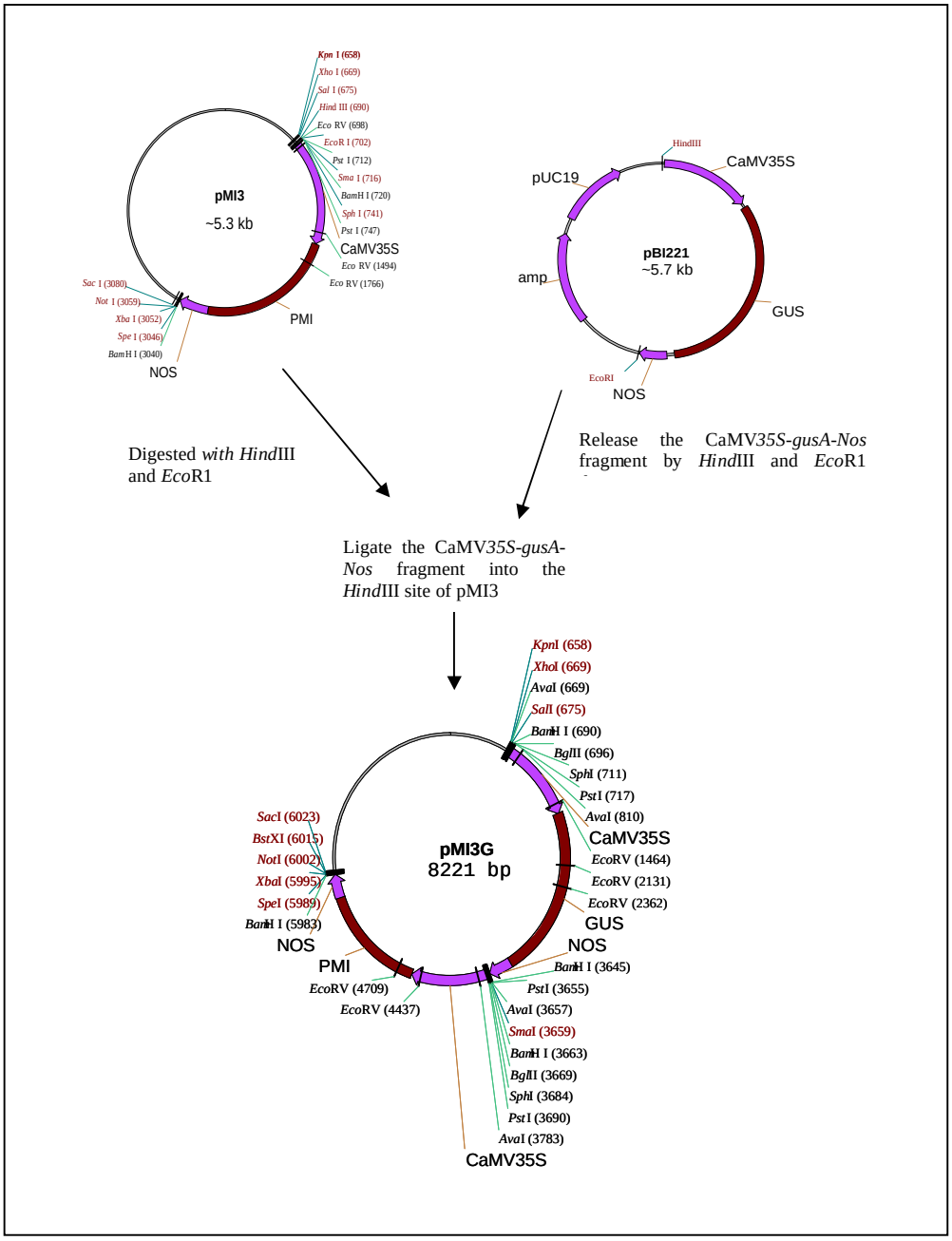

Figure 4.10: Construction of pMI3G. The DNA fragment of CaMV*35S-gusA-Nos* was excised from pBI221 by digestion with *Hind*III and *Eco*R1. Gel purified and ligated to the *Hind*III and *Eco*R1 site of pMI3 to create pMI3G.

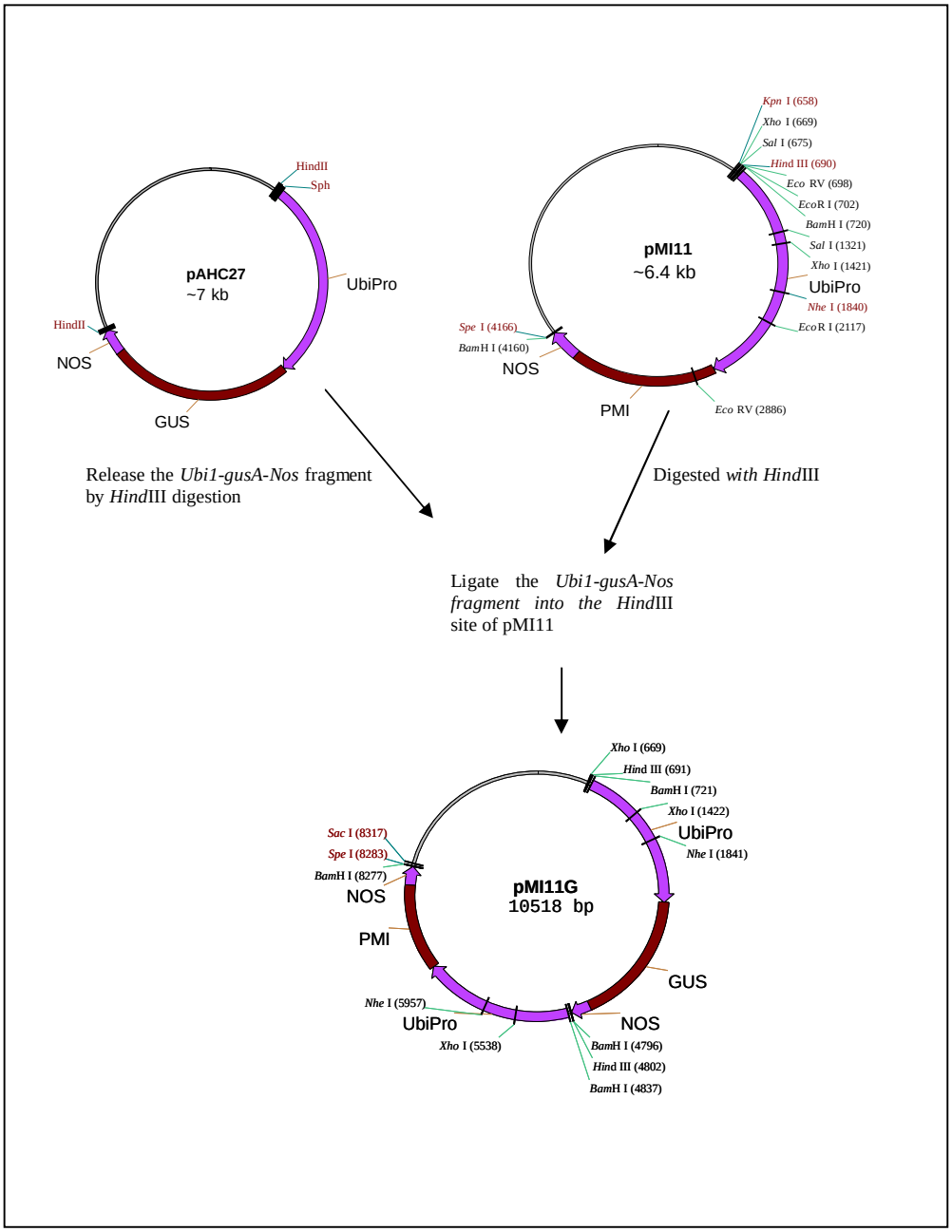

Figure 4.11: Construction of pMI11G. The DNA fragment of *Ubi1-gusA-Nos* was excised from pAHC27 by *Hind*III digestion. Gel purified and ligated to the *Hind*III site of pMI11 to create pMI11G.

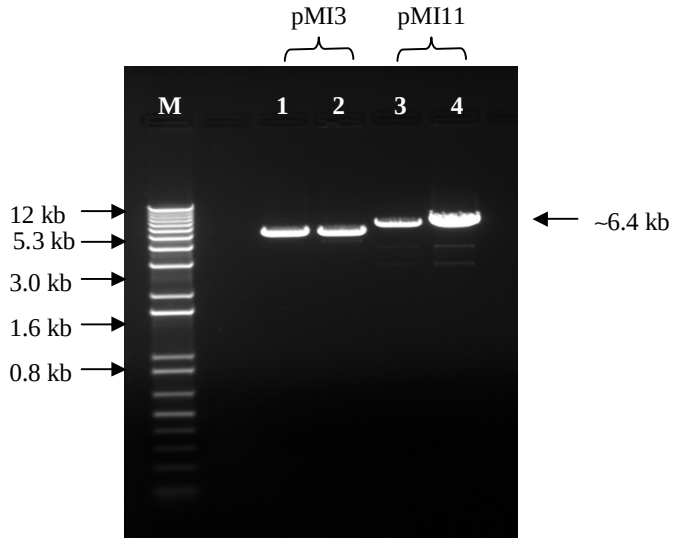

Figure 4.12: Digestion of pMI3 and pMI11 as cloning vectors. Purified DNA was digested with *Hind*III and *Eco*R1 (lanes 1 and 2), and *Hind*III (lanes 3 and 4) and separated on 1% agarose gel in 1XTAE buffer. Arrow indicates the size of fragment, 5.3 kb of pMI3 and 6.4 kb of pMI11.

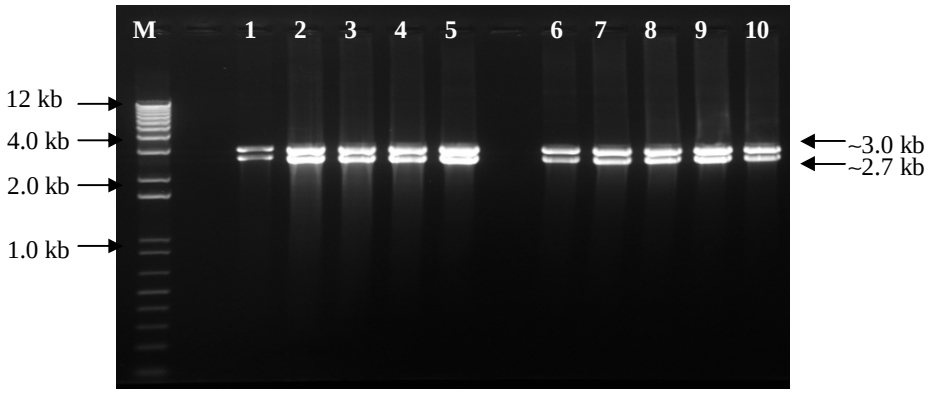

Figure 4.13: Purification of DNA fragment (CaMV*35S-gusA-Nos*) from plasmid pBI221. Purified DNA was digested with *Hind*III and *Eco*RI. Lane M: 1 kb plus DNA ladder; lanes 1-10: Positive clones for CaMV*35S-gusA-Nos* fragment from pBI221. Arrow and size in kb indicate the size of fragment 3.0 kb.

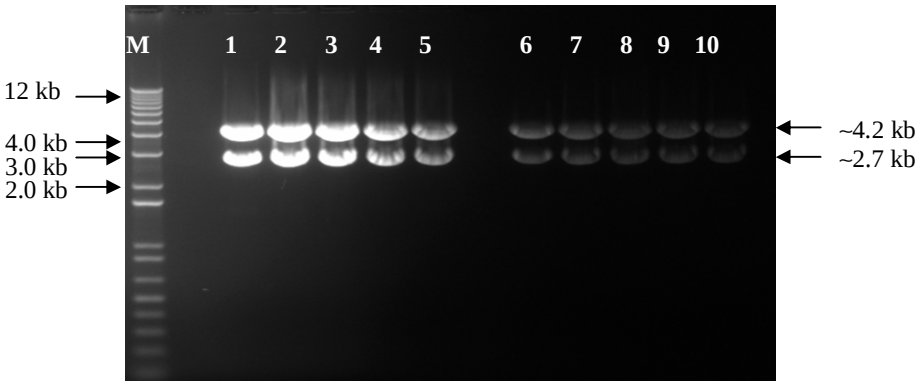

Figure 4.14: Selection of DNA fragment (*Ubi1-gusA-Nos*) from plasmid pAHC27. Purified DNA was digested with *Hind*III. Lane M: 1 kb plus DNA ladder; lanes 1-10: Positive clones for *Ubi1-gusA-Nos* fragment from pAHC27. Arrow and size in kb indicate the size of fragment 4.2 kb.

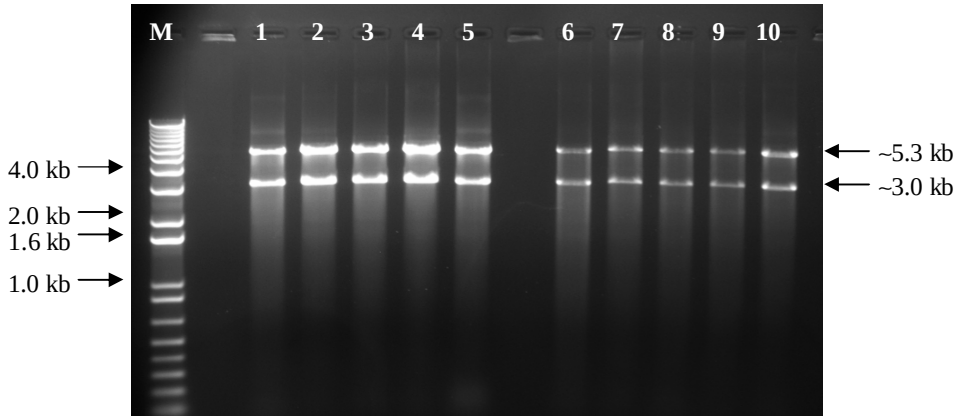

Figure 4.15: Restriction endonuclease analysis of pMI3G. Purified DNA was digested with *Hind*III and *Eco*RI. Lane M: 1 kb plus DNA ladder, lanes 1-10: Positive clones for pBI221 (CaMV*35S-gusA-Nos*) insert, pMI3 vector. Arrow indicates the size of fragment, 3.0 kb and 5.3 kb.

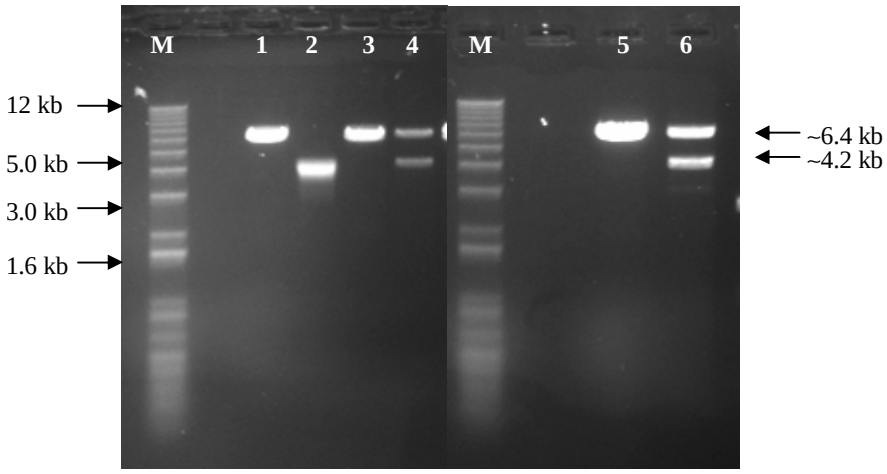

Figure 4.16: Restriction endonuclease analysis of pMI11G. Purified DNA was digested with *Hind*III. Lane M: 1 kb plus DNA ladder, lane 4 and 6: Positive clones for pAHC27 (*Ubi1-gusA-Nos*) insert, pMI11 vector. Arrow indicates the size of fragment, 4.2 kb and 6.4 kb.

4.2 Determination of optimal concentration of mannose as a selection agent

Protocols for positive selection systems are usually based on the ability of transformed plant cells to metabolize compounds that are usually not metabolizable by plants (Joersbo and Okkels, 1996). Thus, the transgenic cells enjoy a metabolic advantage over the untransformed cells that are starved rather than killed. This can be achieved by using a physiologically inert substance as 'selective' agent, which is then converted, due to expression of the appropriate selectable marker gene in transformed cells, into a compound exerting positive effects (Penna et al., 2002). In selection using mannose, mannose-6-phosphate is converted from mannose and accumulates in cells. Concomitant consumption of the mannose-6-phosphate and ATP pools results in severe growth retardation (Ferguson et al., 1958). If other carbohydrate sources are not present in the medium, then only cells that have the ability to convert the derivative selective agent to fructose-6-phosphate could maintain their energy status. Most plant species have been reported not to be able to metabolize mannose (Malca et al., 1967). However,

the level of sensitivity towards mannose is species dependent and has to be empirically determined (Wang et al., 2000).

Therefore, the sensitivity of the oil palm EC and tobacco explants has to be determined before applying the selection systems into the plant transformation work. Specifically, work was carried out to analyse endogenous ability of oil palm oil palm EC and tobacco explants to use mannose as energy source and to identify the selection pressure of mannose on shoot regeneration. The ability of shoot formation was examined by culturing the oil palm EC and tobacco leaf disc on medium containing various concentrations of mannose and sucrose mixture. Medium containing 30 g/l sucrose without mannose was used as positive control. The medium is usually used in regeneration media of oil palm.

4.2.1 Optimal mannose concentration for tobacco transformation as a model plant

For tobacco plants, the results showed that the growth decreased with the increase of mannose concentration (Plate 4.1). The results were similar for plants grown under light and dark conditions. Plants grown under light with full mannose concentration only achieves one third of height of control plant. Plants grown in dark also showed similar response towards different concentration. But difference between plants grown under light and dark conditions was observed. Plants grown under light are more greenish and bushy. Plants grown under dark condition showed chlorosis phenotype. Nevertheless, all plants seem to survive in all treatments. In dark condition where photosynthesis was absent, carbohydrate source was assumed to be minimal. The light conditions applied were also found to influence the sensitivity of the plants towards mannose, demonstrating the influence of the metabolic status of the tissue. This result is in

agreement with the result obtained for tobacco, *Nicotiana tabacum* L. NT1. The group reported an extremely slow growth of tobacco tissues cultured on mannose as the sole carbohydrate source. The tissues doubled every 158 h, compared to 26 h for NT1 cultures on glucose (Barb, 2002).

The result of this study showed that medium containing mannose 30 g/l was suitable to be used in transformation work. The concentration was demonstrated to allow only minimum plant growth. In contrast to other reports, a combination of mannose and sucrose was used, depending on the relative concentrations of sucrose used, the frequency of escapes seemed to increase (He et al., 2006; Jain et al., 2007). 30 g/l mannose equivalent of 30 g/l sucrose was also the standard amount of carbohydrate used in the experiments for the maintenance and selection of oil palm cultures.

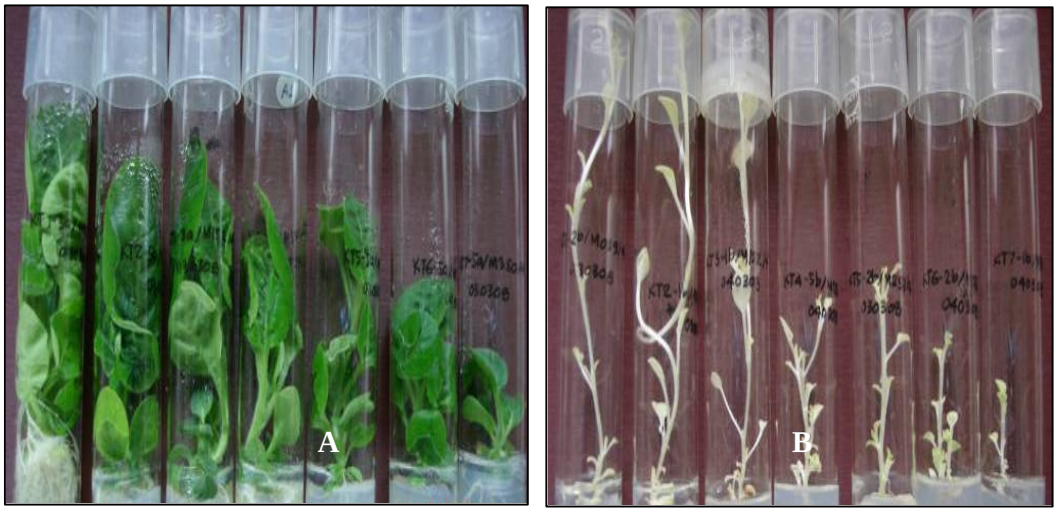

Plate 4.1: Comparison of tobacco plantlets production on media containing various concentrations of mannose: sucrose; from left, 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, 30:0 g/l. Plants incubated in the light (A) and in the dark (B) at 25^{o}C.

4.2.2 Optimal mannose concentration for oil palm transformation

The effects of mannose on oil palm growth were examined by culturing oil palm calli on medium containing various mannose concentrations, ranging from 0.5 to 30 g/l. Figure 4.17 (Refer Appendix G for data tabulation) shows the effect of mannose on plant growth was attained gradually within the initial first three months of treatments. Significant effects could only be seen after four months of culturing. This was based on the least proliferation percentage of the tissues compared to control. The fresh weight ranged from 130 g at 5 g/l mannose to 98 g at 30 g/l mannose. EC weight at the highest concentration of mannose (30 g/l) was only 98 g, which was about half that of the control weight, (154 g). The embryoids did not appear normal. They looked brownish and watery, and had less turgor than embryoids on control medium without mannose. However, the embryoids still survived and were capable of forming shoots. Embryoids formed in media with 30 g/l sucrose (control) were consistently larger than in media with 30 g/l mannose. Adding mannose to the normal culture medium, even at concentrations of up to 3% did not efficiently arrest the growth of the EC. Simultaneously, the numbers of regenerated shoots increased as well, with concomitant inhibition of callus growth. The results of this study showed that the toxic effect of mannose in oil palm increased with the decrease of sucrose concentration.

This difference of average fresh weight of oil palm EC in seven different media with combination mannose and sucrose was confirmed by the statistical Duncan multiple range test. The result is summarized in the form of ANOVA is shown in Table 4.2. The weight of EC grown on 30 g/l mannose was significantly different to EC grown on 30 g/l sucrose (P• 0.05) as determined by ANOVA and the Duncan test.

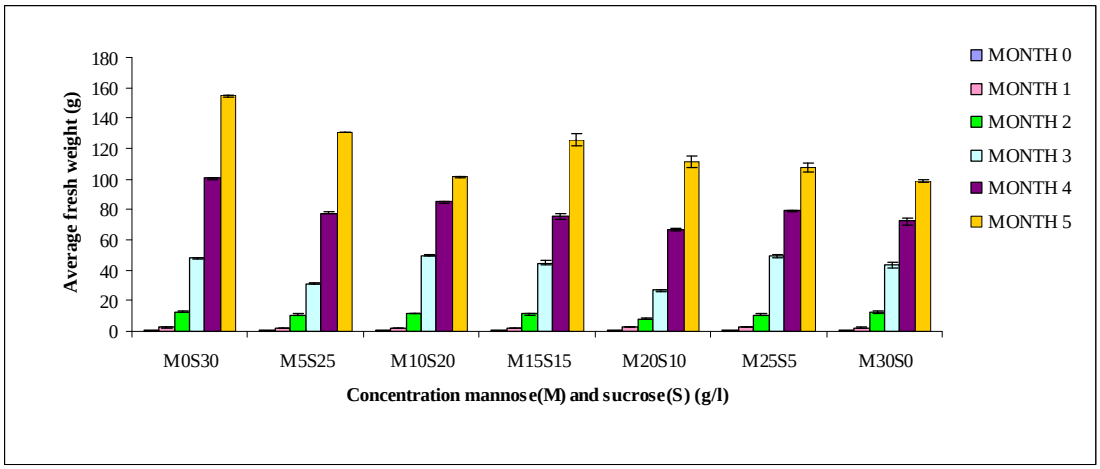

Figure 4.17: Average fresh weight of non-transformed oil palm embryogenic callus against MS medium containing different combination of mannose and sucrose concentrations in monthly incremental for 5 months. The error bars denote the standard error of the mean.

Table 4.2: ANOVA from different combination mannose and sucrose media

	Media [Mannose: Sucrose (g/l)]	**Mean**
1	0:30	154.45 ± 0.29^{e}
2	5:25	130.51 ± 0.26^{d}
3	10:20	101.13 ± 0.32^{ab}
4	15:15	126.03 ± 3.70^{d}
5	20:10	111.48 ± 4.00^{c}
6	25:5	107.12 ± 2.90^{bc}
7	30:0	98.46 ± 0.97^{a}

* 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 replications for five months.

In addition to EC weight, mannose concentrations also seemed to affect the proliferation rate of the calli. The percent proliferation (%) of calli was only at 64% in medium with very high concentration of 30 g/l mannose. This represented a reduction of about 36% compared to control. The % proliferation was shown to steadily increase with the decrease of mannose in the medium. This number was slowly reduced when no sucrose was added to culture medium. Overall, there was a linear decrease in the percentage of plant regeneration from non-transformed EC were grown for five months on various levels of mannose. The growth inhibition observed with mannose treatment was presumably due to the plant cells lacking the enzyme PMI and specific inhibitory effect of mannose, perhaps phosphate and/or ATP depletion rather than carbon depletion (Joersbo et al., 1998).

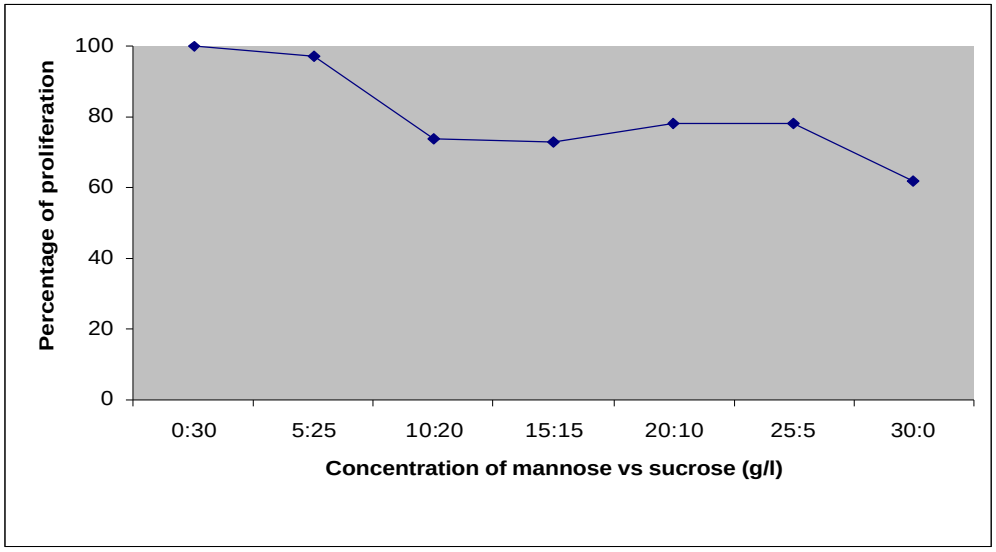

Figure 4.18: Proliferation percentage of oil palm embryogenic callus after 5 months at different concentrations of mannose vs. sucrose (g/l).

The combination of mannose and sucrose produced mostly green callus followed by regeneration of plantlets. Occasionally, the calli did not produce any plantlets. An increase in mannose concentration resulted in a higher number of calli producing shoots. Calli cultured on media containing only mannose as the source of carbohydrate grew normally and produced voluminous green callus, with a recovery of 61%. Media containing sucrose as the sole source of carbohydrate did not produce as much green callus, i.e. 32% (Table 4.3). However, for media containing only mannose, the shoots grew very slowly and eventually turned watery and developed light brown coloration. Shoots also lost vigor during prolonged culture period. Development could potentially be arrested by carbohydrate starvation (Wang et al., 2000) but the plants still survived (Haldrup et al., 1998a).

Table 4.3 also shows the effect of various mannose concentrations on shoot length. The result showed that the shoot height was progressively decreased with the increase of mannose content. Shoots exhibited extremely slow growth on mannose as the sole carbohydrate source; with a 2.5 cm shoot length, 3.5 times less than shoots grown on media with 30 g/l sucrose (8.5 cm) (control). In contrast to selection based on antibiotics or herbicides, which killed non-transformed cells, PMI selection strategy allowed non-transformed cells to survive.

Exp. No. (Man:Suc) (g/l)	No. of explants	Shoot differentiation	% of greenish embryoids	No. of regenerated shoots Min – Max	Length of shoot (cm)
A (0:30)	38		32	10 – 18	8.5
B (5:25)	42		26	13 – 15	7.0
C (10:20)	54		28	5 – 9	6.0
D (15:15)	62		45	5 – 9	5.5
E (20:0)	72		53	8 – 10	5.0
F (25:5)	53		47	3 – 5	4.0
G (30:0)	59		61	4 – 7	2.5

Table 4.3: Effect of various mannose concentrations on shoot formation. The height of shoot decreased with the increase of mannose content in the medium. EC were placed on regeneration medium supplemented with increasing concentrations of mannose and decreasing of sucrose; A (0:30 g/l), B (5:25 g/l), C (10:20 g/l), D (15:15 g/l), E (20:10 g/l), F (25:5 g/l) and G (30:0 g/l). Mean from 3 shoots/condition was measured.

Plate 4.2 shows the shoot formation capability of embryogenic callus in shoot selection medium. The result indicated that shoots exhibited extremely slow growth on mannose as the sole carbohydrate source compared to sucrose. This finding suggested the possibility of little or no PMI activity in the shoots and thus the inability to use mannose as carbon source. When plant cells lacking the enzyme PMI are cultured on medium containing mannose, the cells convert mannose to mannose-6-phosphate, but are unable to isomerize mannose-6-phosphate to fructose-6-phosphate. The accumulation of mannose-6-phosphate inhibited phosphoglucose isomerase, causing a block in glycolysis (Goldsworthy and Street, 1965).

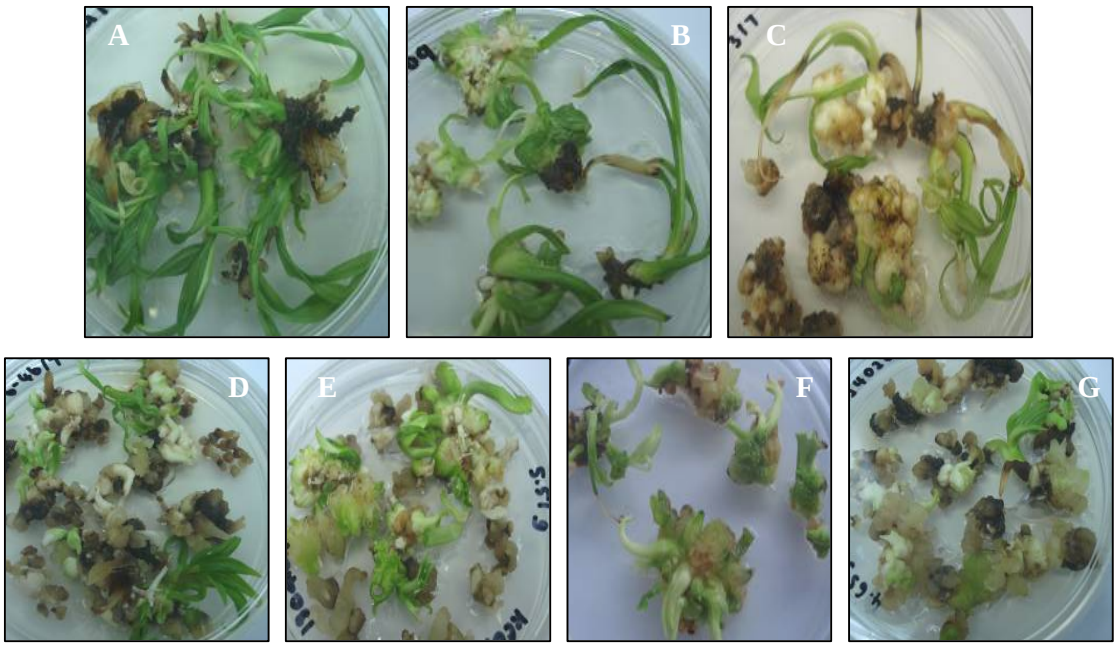

Plate 4.2: Comparison of shoot proliferation from non-transformed oil palm EC on media containing various concentration of mannose: sucrose, A (0:30 g/l), B (5:25 g/l), C (10:20 g/l), D (15:15 g/l), E (20:10 g/l), F (25:5 g/l) and G (30:0 g/l).

The results suggest that the oil palm EC cultures are able to utilize mannose as a carbon source, due to the ability to form shoot. Compared to other species, oil palm can be considered not very sensitive to mannose. The high tolerance may have been due to endogenous PMI expression enabling the plant cells to utilize mannose as a carbohydrate source (Herold and Lewis, 1977) or traces of carbohydrate remaining in the plants. Other reports have shown that other media components, mainly amino acids; glutamine, asparagine and arginine influence the selective effect of mannose which allowed growth of non-transformed cells in presence of mannose (Dekeyser et al., 1989).

The concentration and combination of mannose selection varied for each crop. For example, selection and regeneration of maize and wheat callus were shown effective on 10 g/l mannose and 7 g/l mannose combined with 3 g/l sucrose, respectively (Wright et al., 2001). Negrotto et al., (2000) set a combination of 5g/l sucrose and 10 g/l mannose as the selection index for maize transformation. The mannose concentration of 20 g/l, still allowed low frequency of organogenesis in cassava (Zhang and Puonti-Kaerlas, 2000), while 3 g/l mannose fully inhibited regeneration in sugar beet (Joersbo et al., 1998).

In this study, the optimal selection for oil palm was higher than other crops. This result was in quite similar to result obtained by Kim et al., (2002) on pepper transformation. Shoot formation from cotyledons of pepper was not completely inhibited until mannose concentration reached 50 g/l. To date, this is the highest mannose concentration used for selection of transformed plants. Higher concentrations of sucrose had an additive effect resulting in lower the shoot formation rate of pepper. In this case, mannose itself did not seem to be the sole inhibitor of shoot development. It was shown that sucrose should

also be present in the medium in order to generate a selection pressure (Kim et al., 2002). The difference may be due to different species of plant used and their inherit ability to utilize mannose.

4.3 Biolistic transformation of oil palm with PMI transformation vectors

Optimization of parameters involved in the transformation of oil palm has been achieved previously by Parveez, 1998. In this study, total of 10 plates of embryogenic calli were bombarded with each of the following vectors: pMI3 'CaMV*35S-pmi-Nos'* (TOP1), pMI3G 'CaMV*35S-pmi-Nos*-CaMV*35S-gusA-Nos'* (TOP2), pMI11 '*Ubi1-pmi-Nos'* (TOP3) and pMI11G '*Ubi1-pmi-Nos- Ubi1-gusA-Nos'* (TOP4). Regardless of the transformation method, whether it was biolistic, protoplasts (Wang et al., 2000) *or Agrobacterium,* the PMI selection system has been proven useful for maize and wheat and worked well with other monocots such as rice and barley. Joersbo et al., (1998) and Privalle et al., (2000) have reported the feasibility of PMI system in several dicotyledonous as well.

4.3.1 Selection of transformed callus

In this study, the selection of transformed oil palm EC was carried out with 30 g/l mannose in the selection medium. The 30 g/l concentration of mannose was selected based on the basic medium using sucrose as the carbohydrate source for oil palm regeneration and the result obtained from the preceding experiment. At this concentration, oil palm EC was also shown to be unable to produce normal shoots. Plate 4.3 shows the developmental stages of transformed oil palm tissues from the callus formation until the complete plantlet development. The PMI-transformed and mannose selected EC structures appeared yellow, healthy and grew vigorously on 30 g/l mannose without sucrose. This indicated that PMI-transformed calli can effectively utilize

mannose for its carbon source. The non-tansformed calli selected on mannose grew very slowly, brownish and reduced overall tissue vigor. No significant difference in shoot growth was observed for all the constructs used in the transformation (Plate 4.4). Thus, the deleterious effects of dying cells were to a large extent avoided using mannose selection.

Joersbo et al., 1998 has previously reported that mannose was a better carbohydrate source than sucrose allowing faster growth rates in transgenic sugar beet. Its effect has been correlated to PMI activity. 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 with other sugars such as glucose and fructose, sucrose resulted in a higher transformation frequency in sugar beet. Additional phosphate had a strong positive effect on the transformation frequencies. Therefore, further modifications of the mannose selection protocol might improve the transformation frequencies in oil palm.

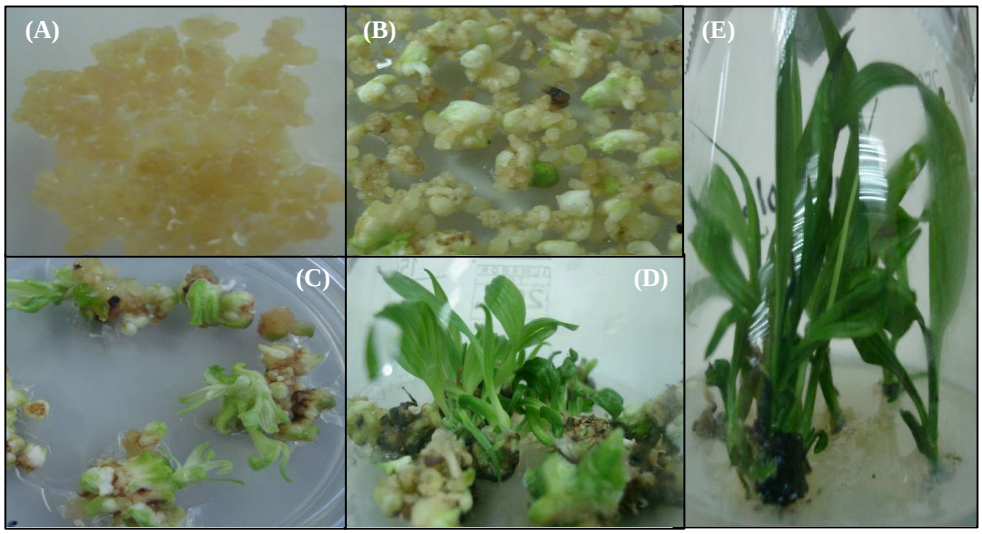

Plate 4.3: Developmental stages of oil palm transformed using *pmi* gene. A: Callus formation stage, B: Embryoid formation stage, C, D: Shoot formation stages and E: Fully developed plantlets.

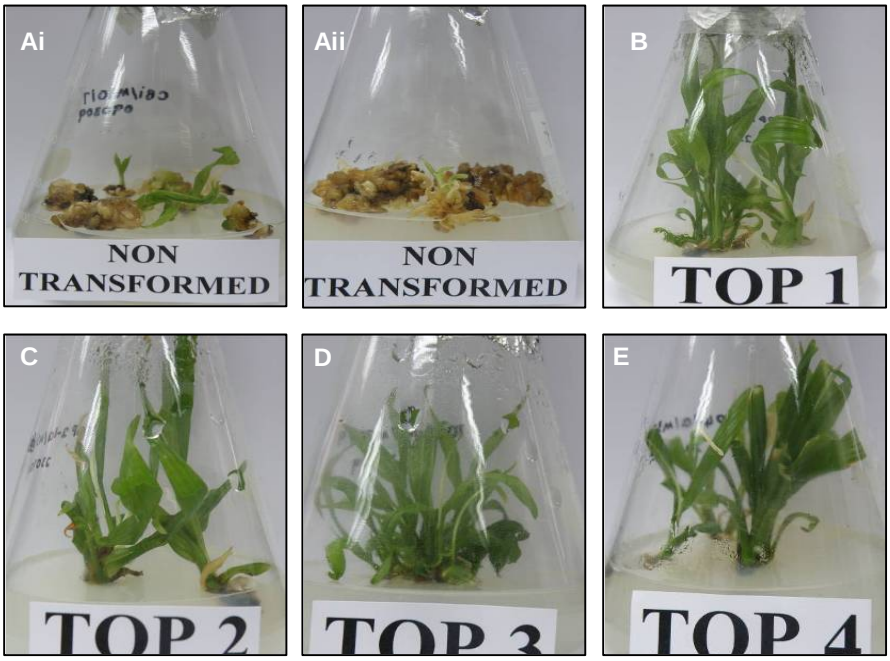

Plate 4.4: Comparison of transgenic oil palm plantlets which carry *pmi* gene on MS media containing 30 g/l mannose. Ai, Aii: Non-bombarded EC, bombarded EC B: TOP1 with pMI3, C: TOP2 with pMI3G, D: TOP3 with pMI11 and E: TOP4 with pMI11G vector.

4.4 Biolistic transformation of tobacco as a model plant

In this study, using the optimized parameters, a total of 20 plates of tobacco leaf discs were bombarded with each of the following vectors: pMI3: 'CaMV*35S-pmi-Nos*' (TOP1), pMI3G: 'CaMV*35S-pmi-Nos*-CaMV*35S-gusA-Nos*' (TOP2), pMI11: '*Ubi1-pmi-Nos*' (TOP3) and pMI11G: '*Ubi1-pmi-Nos-Ubi1-gusA-Nos*' (TOP4). After bombardment, the leaf discs were allowed to proliferate using a selection medium supplemented with 30 g/l mannose as the carbon source and contained 6-Benzylaminopurine (BAP) as growth regulator to induce callus. Cells transformed with the *pmi* gene were able to utilize mannose as a carbon source and grew well. As a result, the transgenic cells enjoy a metabolic advantage compared with the non-transgenic cells that remain unable to metabolize mannose-6-phosphate (Hansen and Wright, 1999). Plate 4.5 shows the developmental stages of transgenic tobacco tissues from callus formation until complete plantlet regeneration. The callus appeared at the cut ends of expanded tobacco leaf disc after a month of bombardment. Then primary shoots appeared in a month and developed rooted plantlet within 2 to 3 months. The transgenic tobacco were maintained and regenerated prior to confirm the gene integrated in the genome and the transgene expression.

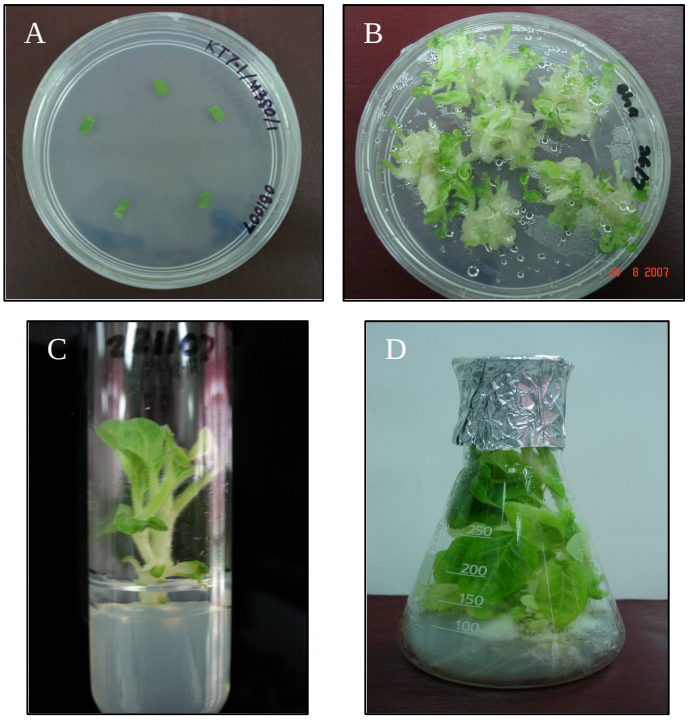

Plate 4.5: Development and regeneration of transgenic tobacco plants. A: Tobacco leaf discs; B: callus and shoot appeared at early stage; C: Shoot development; D: Tobacco plantlet at rooting stage.

4.5 Analysis of transformants

4.5.1 GUS histochemical assay

Two vectors namely, pMI3G and pMI11G carrying the *pmi* and *gusA* reporter genes, were bombarded into oil palm embryogenic calli, embryoids and tobacco leaf discs using biolistic PDS/He 1000 device. The *pmi* gene in the 2 vectors were driven by different promoters, namely CaMV*35S* and *Ubi1*, in pMI3G and pMI11G, respectively. Tissues were also bombarded with gold particles without DNA as a control. Bombarded tissues were histochemically assayed for GUS expression by staining with X-Gluc. This was carried out to ensure that the construct could be used for oil palm transformation. Plate 4.6 showed that blue spots were detected as a result of GUS activity. No GUS 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 GUS expression in all tissues. Less blue spots were detected in tissues carrying *pmi* gene driven by CaMV*35S* promoter compared to *Ubi1* promoter. This is in agreement with result previously reported by Parveez (1998) and Masura (2009). However, the level of GUS expression could be further verified by quantification of β-glucuronidase activity. The use of histochemical GUS assay in comparison studies could be misleading because of leakage of cells which may manifest as a large blue stained area.

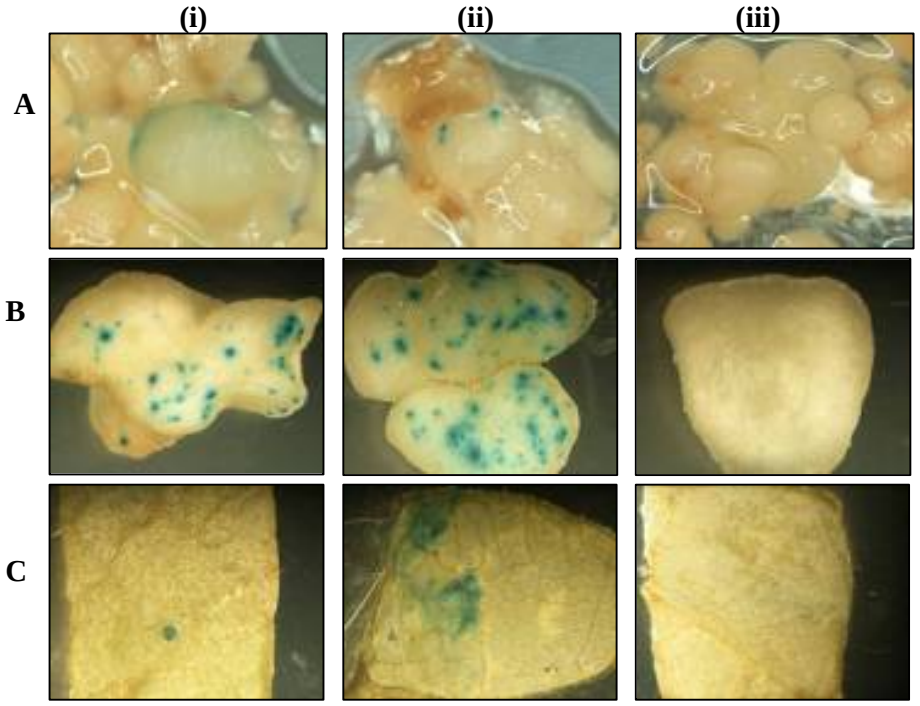

Plate 4.6: Comparison of transient histochemical *gusA* gene expression in; from left, Oil palm embryogenic callus (A) and embryoid (B), tobacco leaf (C) bombarded with plasmids carrying *gusA* gene driven by different promoters. i) pMI3G (CaMV*35S*) and ii) pMI11G (*Ubi1*) and iii) bombarded without DNA.

4.5.2 PCR analysis

Initial screening of putative transgenic plants was conducted at a molecular level using PCR analysis. The analysis was used to examine whether *pmi* gene was present or not in the putative transformed plants. A portion of recovered embryoids was analyzed by PCR. The remaining embryoids were kept for regeneration. Figure 4.19 and 4.20 shows the results of the PCR analysis. A total of 19 out of 21 putative transformed embryoids and 11 out of 17 of transformed plantlets produced the expected amplified DNA size (1.1 kb), demonstrating the presence and presumably the insertion of the *pmi* gene in the genomic DNA. Figure 4.21 shows that approximately 75% of tobacco plantlets (9 out of 12) were positive for *pmi* gene. PCR product was not observed for the untransformed oil palm plant (Lane U), while the positive controls (DNA of transformation vector, Lane P) produced PCR product of the similar size (1.1 kb). The results of the PCR analysis are summarized in Table 4.4 and Table 4.5. The presence of *pmi* gene was verified by using PMI-F and PMI-R primers (Table 3.5). Using PCR analyses, 10% of the embryoids and 35% of the plantlets were found to be negative for the *pmi* gene, and considered as selection escapes. A longer selection period might be required for reducing the escapes. A preliminary transformation efficiency of 79% for oil palm and 75% for tobacco were calculated as the percentage of *pmi* positive confirmed by PCR analysis per total number of sample tested (Table 4.4).

The number transgenic plants regenerated from the *Ubi1* promoter-based vector were not obviously different from the ones transformed with CaMV*35S*-based promoter (Table 4.5, Table 4.6). It could be suggested that the *Ubi1* promoter did not show any difference in the expression of the *pmi* gene in oil palm as compared to the CaMV*35S* 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, CaMV*35S*, in this monocot plant. In contrast, Parveez (1998) and Masura et al., (2010) have shown that the *Ubi1* promoter was better than the CaMV*35S* in driving the transient expression of the *gusA* reporter gene in oil palm embryogenic calli and embryoids. Different promoters were also tested in other transformation experiment using the *pmi* gene. However, no simple correlation between promoter strength and transformation frequency was apparent in sugar beet transformation (Joersbo et al., 2000). The evidence for the beneficial effect of a strong promoter driving the selectable marker gene is not obvious. It has been reported that a relatively weak promoter may be superior (Mengiste et al., 1997) or the promoter strength may appear to be less important (Li et al., 1997).

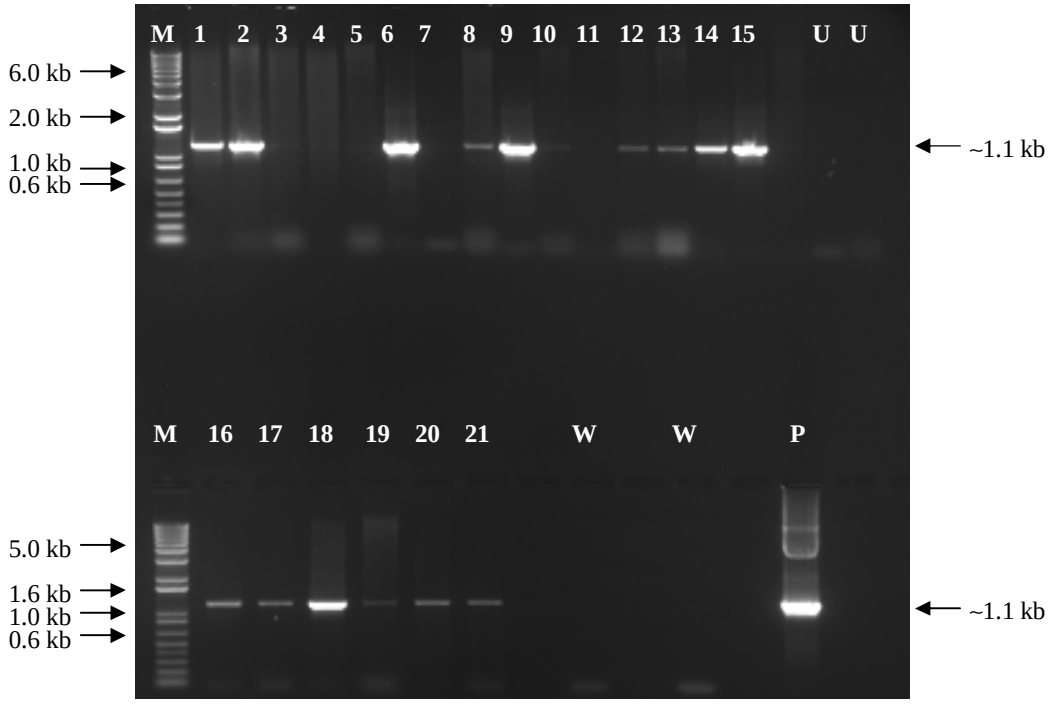

Figure 4.19: PCR analysis of oil palm embryoids using PMIF-PMIR primers to amplify the *pmi* gene. The expected size (1.1 kb) is indicated by an arrow. Lane M = 1 kb plus DNA ladder marker; W = water (negative control); P= pMI3 (positive control); U = Untransformed EC; 1-5 = TOP1; 6-10 = TOP2; 11-15 = TOP3; 16-21 = TOP4.

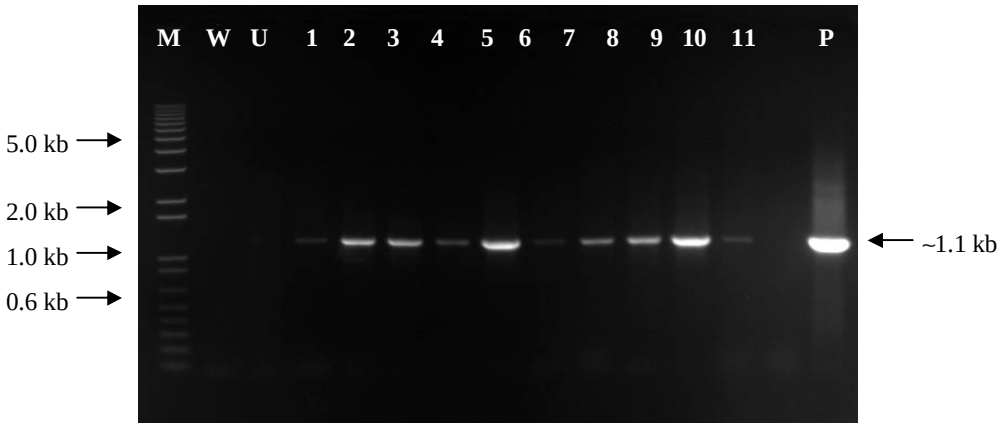

Figure 4.20: PCR analysis of oil palm plantlets using PMIF-PMIR primers to amplify the *pmi* gene. The expected size (1.1 kb) is indicated by an arrow. Lane M = 1 kb plus DNA ladder marker; W = water (negative control); P= pMI3 (positive control); U = Untransformed; 1-2 = TOP1; 3-5 = TOP2; 6-8 = TOP3; 9-11 = TOP4.

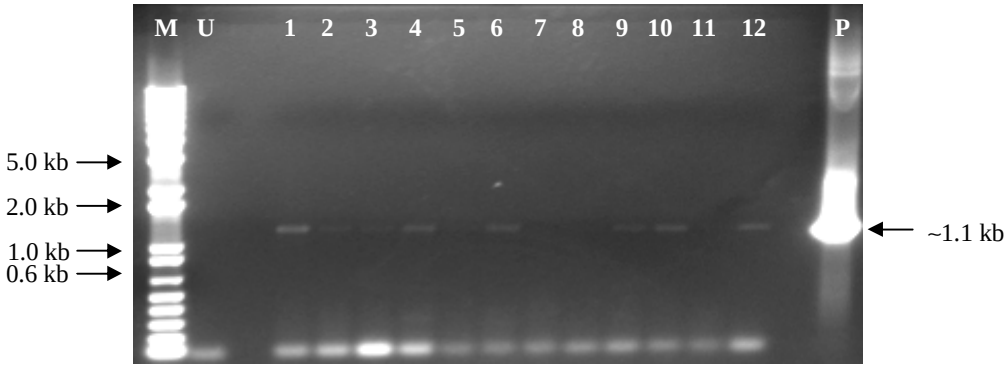

Figure 4.21: PCR analysis of tobacco plantlets using PMIF-PMIR primers to amplify the *pmi* gene. The expected size (1.1 kb) is indicated by an arrow. Lane M = 1kb plus DNA ladder marker; P= pMI3 (positive control); U = Untransformed (negative control); 1-3 = TB1; 4-6 = TB2; 7-9 = TB3; 10-12 = TB4.

Table 4.4: Oil palm and tobacco transformation efficiency carrying *pmi* gene in 30 g/l mannose selection medium.

Plant	Sample	No. of Tested (A)	No. of PCR positive for *pmi* gene (B)	Putative Transformation efficiency (B/A, %)
Oil Palm	Embryoid	21	19	90
	Plantlet	17	11	65
TOTAL		38	30	79
Tobacco	Plantlet	12	9	75

Table 4.5: Presence of *pmi* transgene in regenerated oil palm and tobacco driven by CaMV35S promoter in 30 g/l mannose selection medium as assayed by PCR.

Plant	Sample	No. of Tested		No. of Positive	
		PCR	RT-PCR	PCR	RT-PCR
Oil Palm	Embryoid	10	8	9	8
	Plantlet	8	-	5	-
Tobacco	Plantlet	6	-	4	-

- not done due to unavailability of samples

Table 4.6: Presence of *pmi* transgene in regenerated oil palm and tobacco driven by *Ubi1* promoter in 30 g/l mannose selection medium as assayed by PCR.

Plant	Sample	No. of Tested		No. of Positive	
		PCR	RT-PCR	PCR	RT-PCR
Oil Palm	Embryoid	11	6	10	6
	Plantlet	9	-	6	-
Tobacco	Plantlet	6	-	5	-

- not done due to unavailability of samples

4.5.3 Sequencing and identification of PMI

In order to verify the identity of the amplified fragment, the 1.1 kb PCR product was cloned and sequenced. The sequence obtained was subjected to BLAST analysis against deposited sequences in the GenBank database (Figure 4.22). The BLAST results indicated that the DNA sequence of the amplified fragment of oil palm and tobacco *pmi* is highly similar to their corresponding *pmi* gene from *Escherichia coli* (Genbank accession no: M15380) about 98%.

```
                                1                                                50

E. guineensis pmi     (1) ----------------------------------------CAGCAAAAC
       E.coli pmi     (1) ATGCAAAAACTCATTAACTCAGTGCAAAACTATGCCTGGGGCAGCAAAAC
  N. tabaccum pmi     (1) ----------------------------------------CAGCAAAAC
       Consensus      (1)                                            CAGCAAAAC
                          51                                                100
E. guineensis pmi    (10) GGCGTTGACTG-AACTTTATGGTATGG-AAAATCCGTCCAGCCAGCCGAT
       E.coli pmi    (51) GGCGTTGACTG-AACTTTATGGTATGG-AAAATCCGTCCAGCCAGCCGAT
  N. tabaccum pmi    (10) GGCGTTGACTG-AACTTTATGGTATGG-AAAATCCGTCCAGCCAGCCGAT
       Consensus     (51) GGCGTTGACTG AACTTTATGGTATGG AAAATCCGTCCAGCCAGCCGAT
                         101                                                150
E. guineensis pmi    (60) GGCCGAGCTGTGGATGGGCGCACATCCGAAAAGCAGTTCACGAGTGCAGA
       E.coli pmi    (99) GGCCGAGCTGTGGATGGGCGCACATCCGAAAAGCAGTTCACGAGTGCAGA
  N. tabaccum pmi    (58) GGCCGAGCTGTGGATGGGCGCACATCCGAAAAGCAGTTCACGAGTGCAGA
       Consensus    (101) GGCCGAGCTGTGGATGGGCGCACATCCGAAAAGCAGTTCACGAGTGCAGA
                         151                                                200
E. guineensis pmi   (110) ATGCCGCCGGAGATATCGTTTCACTGCGTGATGTGATTGAGAGTGATAAA
       E.coli pmi   (149) ATGCCGCCGGAGATATCGTTTCACTGCGTGATGTGATTGAGAGTGATAAA
  N. tabaccum pmi   (108) ATGCCGCCGGAGATATCGTTTCACTGCGTGATGTGATTGAGAGTGATAAA
       Consensus    (151) ATGCCGCCGGAGATATCGTTTCACTGCGTGATGTGATTGAGAGTGATAAA
                         201                                                250
E. guineensis pmi   (160) TCGACTCTGCTCGGAGAGGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
       E.coli pmi   (199) TCGACTCTGCTCGGAGAGGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
  N. tabaccum pmi   (158) TCGACTCTGCTCGGAGAGGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
       Consensus    (201) TCGACTCTGCTCGGAGAGGCCGTTGCCAAACGCTTTGGCGAACTGCCTTT
                         251                                                300
E. guineensis pmi   (210) CCTGTTCAAAGTATTATGCGCAGCACAGCCACTCTCCATTCAGGTTCATC
       E.coli pmi   (249) CCTGTTCAAAGTATTATGCGCAGCACAGCCACTCTCCATTCAGGTTCATC
  N. tabaccum pmi   (208) CCTGTTCAAAGTATTATGCGCAGCACAGCCACTCTCCATTCAGGTTCATC
       Consensus    (251) CCTGTTCAAAGTATTATGCGCAGCACAGCCACTCTCCATTCAGGTTCATC
                         301                                                350
E. guineensis pmi   (260) CAAACAAACACAATTCTGAAATCGGTTTTGCCAAAGAAAATGCCGCAGGT
       E.coli pmi   (299) CAAACAAACACAATTCTGAAATCGGTTTTGCCAAAGAAAATGCCGCAGGT
  N. tabaccum pmi   (258) CAAACAAACACAATTCTGAAATCGGTTTTGCCAAAGAAAATGCCGCAGGT
       Consensus    (301) CAAACAAACACAATTCTGAAATCGGTTTTGCCAAAGAAAATGCCGCAGGT
                         351                                                400
E. guineensis pmi   (310) ATCCCGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAACCACAAGCC
       E.coli pmi   (349) ATCCCGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAACCACAAGCC
  N. tabaccum pmi   (308) ATCCCGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAACCACAAGCC
       Consensus    (351) ATCCCGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAACCACAAGCC
                         401                                                450
E. guineensis pmi   (360) GGAGCTGGTTTTTGCGCTGACGCCTTTCCTTGCGATGAACGCGTTTCGTG
       E.coli pmi   (399) GGAGCTGGTTTTTGCGCTGACGCCTTTCCTTGCGATGAACGCGTTTCGTG
  N. tabaccum pmi   (358) GGAGCTGGTTTTTGCGCTGACGCCTTTCCTTGCGATGAACGCGTTTCGTG
       Consensus    (401) GGAGCTGGTTTTTGCGCTGACGCCTTTCCTTGCGATGAACGCGTTTCGTG
                         451                                                500
E. guineensis pmi   (410) AATTTTCCGAGATTGTCTCCCTACTCCAGCCGGTCGCAGGTGCACATCCG
       E.coli pmi   (449) AATTTTCCGAGATTGTCTCCCTACTCCAGCCGGTCGCAGGTGCACATCCG
  N. tabaccum pmi   (408) AATTTTCCGAGATTGTCTCCCTACTCCAGCCGGTCGCAGGTGCACATCCG
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        Consensus   (451) AATTTTCCGAGATTGTCTCCCTACTCCAGCCGGTCGCAGGTGCACATCCG
                          501                                             550
E. guineensis pmi   (460) GCGATTGCTCACTTTTTACAACAGCCTGATGCCGAACGTTTAAGCGAACT
      E.coli pmi    (499) GCGATTGCTCACTTTTTACAACAGCCTGATGCCGAACGTTTAAGCGAACT
  N. tabaccum pmi   (458) GCGATTGCTCACTTTTTACAACAGCCTGATGCCGAACGTTTAAGCGAACT
        Consensus   (501) GCGATTGCTCACTTTTTACAACAGCCTGATGCCGAACGTTTAAGCGAACT
                          551                                             600
E. guineensis pmi   (510) GTTCGCCAGCCTGTTGAATATGCAGGGTGAAGAAAAATCCTGCGCGCTGG
      E.coli pmi    (549) GTTCGCCAGCCTGTTGAATATGCAGGGTGAAGAAAAATCCCGCGCGCTGG
  N. tabaccum pmi   (508) GTTCGCCAGCCTGTTGAATATGCAGGGTGAAGAAAAATCCTGCGCGCTGG
        Consensus   (551) GTTCGCCAGCCTGTTGAATATGCAGGGTGAAGAAAAATCCTGCGCGCTGG
                          601                                             650
E. guineensis pmi   (560) CGATTTTAAAATCGGCCCTCGATAGCCAGCAGGGTGAACCGTGGCAAACG
      E.coli pmi    (599) CGATTTTAAAATCGGCCCTCGATAGCCAGCAGGGTGAACCGTGGCAAACG
  N. tabaccum pmi   (558) CGATTTTAAAATCGGCCCTCGATAGCCAGCAGGGTGAACCGTGGCAAACG
        Consensus   (601) CGATTTTAAAATCGGCCCTCGATAGCCAGCAGGGTGAACCGTGGCAAACG
                          651                                             700
E. guineensis pmi   (610) ATTCGTTTAATTTCTGAATTTTACCCGGAAGACAGCGGTCTGTTCTCCCC
      E.coli pmi    (649) ATTCGTTTAATTTCTGAATTTTACCCGGAAGACAGCGGTCTGTTCTCCCC
  N. tabaccum pmi   (608) ATTCGTTTAATTTCTGAATTTTACCCGGAAGACAGCGGTCTGTTCTCCCC
        Consensus   (651) ATTCGTTTAATTTCTGAATTTTACCCGGAAGACAGCGGTCTGTTCTCCCC
                          701                                             750
E. guineensis pmi   (660) GCTATTGCTGAATGTGGTGAAATTGAACCCTGGCGAAGCGATGTTCCTGT
      E.coli pmi    (699) GCTATTGCTGAATGTGGTGAAATTGAACCCTGGCGAAGCGATGTTCCTGT
  N. tabaccum pmi   (658) GCTATTGCTGAATGTGGTGAAATTGAACCCTGGCGAAGCGATGTTCCTGT
        Consensus   (701) GCTATTGCTGAATGTGGTGAAATTGAACCCTGGCGAAGCGATGTTCCTGT
                          751                                             800
E. guineensis pmi   (710) TCGCTGAAACACCGCACGCTTACCTGCAAGGCGTGGCGCTGGAAGTGATG
      E.coli pmi    (749) TCGCTGAAACACCGCACGCTTACCTGCAAGGCGTGGCGCTGGAAGTGATG
  N. tabaccum pmi   (708) TCGCTGAAACACCGCACGCTTACCTGCAAGGCGTGGCGCTGGAAGTGATG
        Consensus   (751) TCGCTGAAACACCGCACGCTTACCTGCAAGGCGTGGCGCTGGAAGTGATG
                          801                                             850
E. guineensis pmi   (760) GCAAACTCCGATAACGTGCTGCGTGCGGGTCTGACGCCTAAATACATTGA
      E.coli pmi    (799) GCAAACTCCGATAACGTGCTGCGTGCGGGTCTGACGCCTAAATACATTGA
  N. tabaccum pmi   (758) GCAAACTCCGATAACGTGCTGCGTGCGGGTCTGACGCCTAAATACATTGA
        Consensus   (801) GCAAACTCCGATAACGTGCTGCGTGCGGGTCTGACGCCTAAATACATTGA
                          851                                             900
E. guineensis pmi   (810) TATTCCGGAACTGGTTGCCAATGTGAAATTCGAAGCCAAACCGGCTAACC
      E.coli pmi    (849) TATTCCGGAACTGGTTGCCAATGTGAAATTCGAAGCCAAACCGGCTAACC
  N. tabaccum pmi   (808) TATTCCGGAACTGGTTGCCAATGTGAAATTCGAAGCCAAACCGGCTAACC
        Consensus   (851) TATTCCGGAACTGGTTGCCAATGTGAAATTCGAAGCCAAACCGGCTAACC
                          901                                             950
E. guineensis pmi   (860) AGTTGTTGACCCAGCCGGTGAAACAAGGTGCAGAACTGGACTTCCCGATT
      E.coli pmi    (899) AGTTGTTGACCCAGCCGGTGAAACAAGGTGCAGAACTGGACTTCCCGATT
  N. tabaccum pmi   (858) AGTTGTTGACCCAGCCGGTGAAACAAGGTGCAGAACTGGACTTCCCGATT
        Consensus   (901) AGTTGTTGACCCAGCCGGTGAAACAAGGTGCAGAACTGGACTTCCCGATT
                          951                                            1000
E. guineensis pmi   (910) CCAGTGGATGATTTTGCCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
      E.coli pmi    (949) CCAGTGGATGATTTTGCCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
  N. tabaccum pmi   (908) CCAGTGGATGATTTTGCCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
        Consensus   (951) CCAGTGGATGATTTTGCCTTCTCGCTGCATGACCTTAGTGATAAAGAAAC
                         1001                                            1050
E. guineensis pmi   (960) CACCATTAGCCAGCAGAGTGCCGCCATTTTGTTCTGCGTCGAAGGCGATG
      E.coli pmi    (999) CACCATTAGCCAGCAGAGTGCCGCCATTTTGTTCTGCGTCGAAGGCGATG
  N. tabaccum pmi   (958) CACCATTAGCCAGCAGAGTGCCGCCATTTTGTTCTGCGTCGAAGGCGATG
        Consensus  (1001) CACCATTAGCCAGCAGAGTGCCGCCATTTTGTTCTGCGTCGAAGGCGATG
                         1051                                            1100
E. guineensis pmi  (1010) CAACGTTGTGGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGGTGAATCA
      E.coli pmi   (1049) CAACGTTGTGGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGGTGAATCA
  N. tabaccum pmi  (1008) CAACGTTGTGGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGGTGAATCA
        Consensus  (1051) CAACGTTGTGGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGGTGAATCA
```

```
                          1101                                                1150
E. guineensis pmi  (1060) GCGTTTATTGCCGCCAACGAATCACCGGTGACTGTCAAAG----------
       E.coli pmi  (1099) GCGTTTATTGCCGCCAACGAATCACCGGTGACTGTCAAAGGCCACGGCCG
  N. tabaccum pmi  (1058) GCGTTTATTGCCGCCAACGAATCACCGGTGACTGTCAAAG----------
         Consensus  (1101) GCGTTTATTGCCGCCAACGAATCACCGGTGACTGTCAAAG
                           1151                    1178
E. guineensis pmi  (1099) ----------------------------
       E.coli pmi  (1149) TTTAGCGCGTGTTTACAACAAGCTGTAA
  N. tabaccum pmi  (1098) ----------------------------
         Consensus  (1151)
```

Figure 4.22: Comparison of DNA sequence between oil palm PMI, tobacco PMI and its corresponding *pmi* gene of *E. coli.*

4.5.4 RT-PCR analysis

In order to study the accumulation of *pmi* gene transcripts in oil palm, RT-PCR using reverse transcribed total RNA was performed to confirm PMI expression in the transgenic plants. Total RNA was isolated from 14 samples of transgenic oil palm embryoids using modified Doyle and Doyle (1987) method. The integrity of isolated RNA samples was verified by agarose gel electrophoresis. Results showed that 28S rRNA was more abundant than18S rRNA in all samples, indicating good quality of the extracted samples (Figure 4.23). The reverse transcripts were used in PCR amplification using primer set of PMI-F and PMI-R. In addition, the ribosomal 18S RNA primer was used as the endogenous control for RNA quantification (Figure 4.24). Results showed that the expected 1.1 kb fragment for *pmi* gene was obtained from all 14 transgenic plants generated using pMI3 (TOP1), pMI3G (TOP2), pMI11 (TOP3) and pMI11G (TOP4) constructs. The products obtained (bands) were faint and absent in the non-transgenic plants (Figure 4.25). This observation indicated that the *pmi* transgenes were transcriptionally active in the transgenic plants. The results of the RT-PCR analysis are summarized in Table 4.5 and Table 4.6.

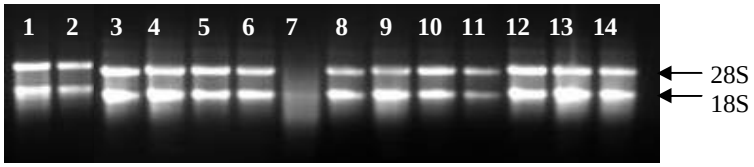

Figure 4.23: Total RNA isolated from oil palm embryoids. Each lane was loaded with 5 ug of total RNA. The intact RNA (28S and 18S) was observed. Lanes; 1-3, TOP1; 4-8, TOP2; 9-11, TOP3; 12-14, TOP4.

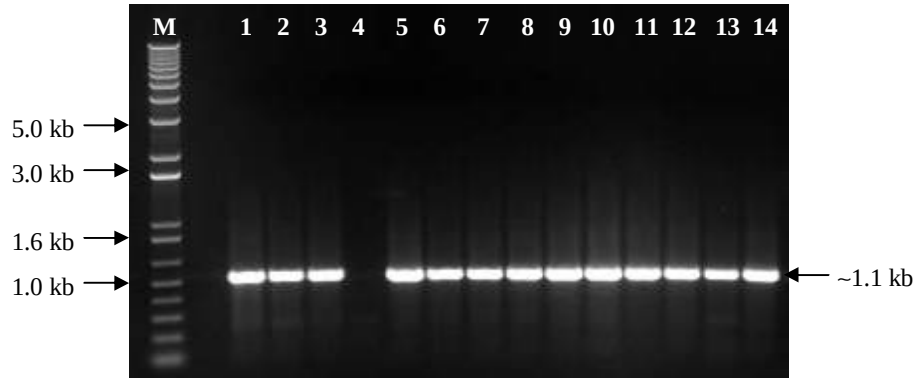

Figure 4.24: RT-PCR analysis from total RNA extracted from oil palm embryoids for the detection of a 1.1 kb 18S fragment. Lane M = 1kb plus DNA ladder marker; 1-3 = TOP1; 4-8 = TOP2; 9-11 = TOP3; 12-14 = TOP4.

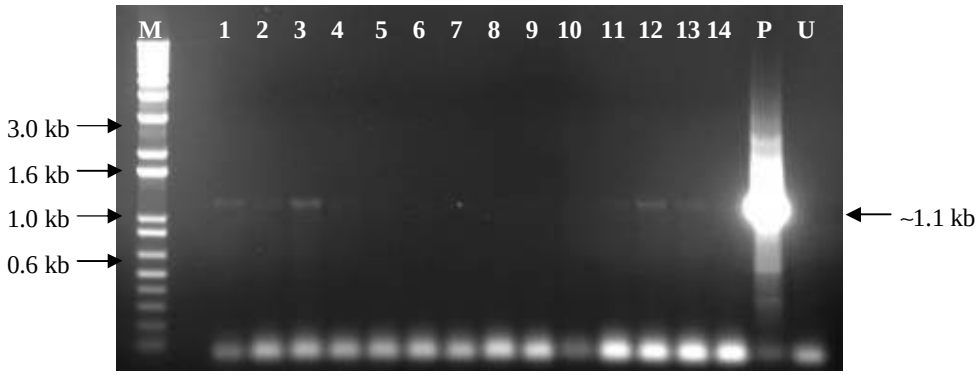

Figure 4.25: RT-PCR product from total RNA extracted from oil palm embryoids. The expected size (1.1 kb) is indicated by an arrow. Lane M = 1kb plus DNA ladder marker; U = non-transformed (negative control); P= pMI3 (positive control); 1-3 = TOP1; 4-8 = TOP2; 9-11 = TOP3; 12-14 = TOP4.

4.6 Proposed future work

For future studies on oil palm transformation, direct comparison between mannose and antibiotic or herbicide selections need to be carried out. Dose response tests of mannose selection with dosage higher than 30 g/l of mannose maybe required to totally inhibit shoot organogenesis. The new condition could result in better transformation efficiencies. Subsequently, this will prove that transformation efficiency is higher using positive selection compared to negative selection.

Further analysis using molecular approach such as Southern hybridization will definitely be required to prove the integration of transgenes in the plant genome as well as to determine the transgene copy numbers. The presence of protein could be verified through either Western analysis or Elisa immunoassay.

Finally, it is also necessary to design constructs containing *pmi* gene together with a gene of interest and to transform into plant cells without using negative selection marker systems such as antibiotic or herbicide resistance. Results from such work will definitely prove the usefulness of the PMI selection system in oil palm transformation.

CHAPTER 5

CONCLUSION

A new selection procedure has been developed for oil palm (*E. guineensis* Jacq) transformation. The selection system utilized the *pmi* gene from *E. coli* and mannose as its selection agent. The usage of mannose as metabolizable sugar in this positive selection system eliminated the problem of using negative selection systems, such as herbicides or antibiotic resistance genes.

In order to assess the efficiency of mannose selection in oil palm transformation, the expression vectors containing *pmi* gene were constructed and bombarded into embryogenic calli. Mannose, of the concentration 3% was used as selection agent and served as the only carbohydrate source in oil palm cultures.

Determination of optimal concentration of mannose as selective agent on oil palm embryogenic calli indicated that the effect of mannose on plant growth was attained gradually within the initial first three months of treatments. Significant effects could only be seen after four months of culturing. The optimal selection of mannose was found at 30 g/l allowing the explants to induce shoots. At this concentration, the explants were shown to be able to regenerate into shoots.

Molecular analyses were performed to confirm the transgenic status of the transgenic embryoids and plantlets. A histochemical assay for GUS was used for early identification of gene transfer events and to ensure that the constructs could be used for oil palm transformation. GUS activity was observed in the transformed tissues. The results suggested the presence of the introduced gene and the transgene activity in

transgenic oil palm produced. Transgenic plants were confirmed by PCR and the transgene expression was examined using RT-PCR analysis. Based on the positive results from the PCR screening, a preliminary transformation efficiency of 79% for oil palm and 75% for tobacco were calculated. The transgenic plant events regenerated from the *Ubi1* promoter-based vector was not much different from the number from the CaMV*35S*-based promoter. It could be suggested that the *Ubi1* promoter did not show any difference in the expression of the *pmi* gene in oil palm as compared to the CaMV*35S* promoter. The transgene insertion verification was confirmed by the high identity (98%) observed between the amplified fragment corresponding *pmi* gene from *Escherichia coli.*

The utilization of tobacco as a model plant was proven to be successful. Leaf explants were bombarded and selected on mannose. The explants were able to regenerate and produce transgenic plants. Transgenic plants were confirmed by PCR analysis, GUS histochemical assay and 98% identity with *pmi* gene to the PMI *Escherichia coli* sequence.

However, further analyses such as Southern and Western analyses are certainly required to further prove the transgenic status of the transformants. Nevertheless, the results of this study indicated that the mannose selection system can be used in oil palm transformation. The use of the system will ensure a more acceptable transgenic oil palm in the future.