Development of a Protoplast-based Transformation System for Genetic Engineering of Oil Palm

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

vorgelegt von

Master of Science

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Tag der mündlichen Prüfung:

09.07.2013

This thesis is dedicated to my loving father, mother and family

SUMMARY

The major aim of the thesis was to develop the prerequisites for efficient genetic engineering of oil palm by DNA microinjection with the long-term objective to generate transgenic oil palm producing recombinant proteins, PIPP (a chimeric antibody against human chorionic gonadotropin; hCG), D12 (a human antibody against dental carries) and HSA (human serum albumin). The products will be synthesized in the leaf, mesocarp and kernel tissues of oil palm with the respects of plants must be stable and free from selectable marker. To achieve this, the constructs of PIPP, D12 and HSA genes, which were driven either by the promoter of LSP, MSP or KSP were successful constructed and their functionality was demonstrated in tobacco plants.

To implement the oil palm protoplasts as starting material for the development of stable transgenic oil palms via DNA microinjection, the regeneration of true plants from protoplasts is a mandatory. Therefore, an improved protocol for the efficient isolation of high-quality protoplasts from oil palm suspension cultures was established. Subsequently, for the first time true oil palms were successfully regenerated from oil palm protoplasts by using optimal parameters. Nearly 14-17 months after protoplasts were isolated; true plants were generated using agarose bead culture. Following the success in regeneration of plants from protoplasts, the objective of this project became clearly to be achieved in the future when the protoplasts were used for PEG-mediated transient gene expression, and further used in the stable gene expression via DNA microinjection. The efficient and reliable protocol for PEG mediated transformation of oil palm protoplasts was developed by determing and validating the optimal parameters like heat shock treatment, the amount of DNA, PEG and magnesium chloride concentrations, and the procedure to transfect the protoplasts. As the main objective of this study, the transgenic microcalli of oil palm were successful generated from protoplasts transformed by DNA microinjection within 6 months. More conclusive results will be obtained when small plantlets are produced and analyzed.

ACKNOWLEDGEMENTS

First at all I wish to express my sincere thanks to Prof. Dr. Rainer Fisher and Prof. Dr. Dirk Pruefer for giving me the opportunity to obtain my PhD in this collaborative project.

I want to express my great gratitude to my main supervisor, Prof. Dr. Dirk Pruefer, for his great support, encouragements, friendship, and insight throughout the course of my study at Fraunhofer-IME (Dependence Muenster), Germany. Without his thoughtful arrangements, instructions and help, it would be impossible for me to complete this study.

I sincerely thank Dr. Gundula Noll (University of Muenster, Germany) for her direct daily supervision and also for her brilliant ideas, kind advises and discussions during this study. My thanks also go to Dr. Ahmad Parveez Ghulam Kadir (Head of Functional Biotechnology unit, MPOB), for his advice, comments and guidance not only in science but also in different aspects of life whenever sought.

I would like to present honest thanks to the Malaysian Palm Oil Board (MPOB) for financial support in the course of MPOB and IME Board approved collaborative project "Establishment of Transgenic Oil Palm with High Added Value for commercial exploitation" (T0003050000-RB01-J). Special thanks to Datuk Dr. Choo Yuen May, (Director General MPOB), Dr. Ahmad Kushairi (Deputy Director General MPOB) and Dr. Ravigadevi (Director ABBC) for allowing me to completed my thesis. My thanks also go to Pn. Ruba'ah Masri and Pn Suraya Harun (Training Unit, MPOB).

Special thanks extended to the members of AG Prof. Dirk Pruefer (IME, Muenster, Germany) and Transgenic Technology Group (MPOB) for their assistance. Finally, I wish to express my deepest gratitude and appreciation to my family, especially to my father (Mat Yunus Abas) and mother (Adiyah Hj Mokhtar), for their continuous long-distance encouragement and support from Kumai, Pahang. To my wife, Dayang Izawati, thank you for your love and encouragement during my study.

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ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	2-γ-Dimethylallylamino-purine
35S	cauliflower mosaic virus 35S
35ST	gene for terminator of 35S
5-CFDA,AM	5-CarboxyFluorescein Diacetate, AcetoxyMethyl ester
AA	Ascorbic Acid
AC	Activated Charcoal
$AgNO_3$	Silver nitrate
BA	6-Benzyl Aminopurine
cDNA	complementary DNA
CE	Compact Embryogenic callus
CFDV	Coconut Foliar Decay Virus
CLSM	Confocal Laser Scanning Microscopy
D12	Anti-caries antibody D12
DNA	Deoxyribo Nucleic Acid
dNTP	deoxy Nicotinamide Triphosphate
ER	Endoplasmic Reticulum
FE	Friable Embryogenic callus
fwt	fresh weight
GA3	Gibberellic acid A3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
Hc	Heavy-chain
hrGFP	humanized renilla Green Fluorescent Protein
HSA	Human Serum Albumin
hyg	hygromycin
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
IME	Institute for Molecular Biology and Applied Ecology
KCl	Potassium chloride

KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium nitrate
KSP	Kernel-Specific Promoter
Lc	Light-chain
LHCB	Light-Harvesting Chlorophyll a/b Binding protein
LSP	Leaf-Specific Promoter
$MgCl_2$	Magnesium chloride
MPOB	Malaysian Palm Oil Board
MS media	Murashige and Skoog media
MSP	Mesocarp-Specific Promoter
MT3-A	fruit-specific type 3 metallothionein-like gene
NAA	Naphthalene Acetic Acid
NH_4	Ammonium
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate
$NiCl_2.6H_2O$	Nikel chloride
NO_3	Nitrate
pBS	pBluescriptII KS^+
PCR	Polymerase Chain Reaction
PEG	Poly Ethlyene Glycol
PGRs	Plant Growth Regulators
PIPP	hCG-specific antibody PIPP
RNA	Ribonucleic Acid
rpm	round per minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
T-DNA	transferable-DNA
Ubi	Maize ubiquitin promoter
v/v	volume per volume
w/v	weight per volume
Zea	Zeatin

I INTRODUCTION

I.1 Reasons for oil palm genetic engineering

I.1.1 Limited land resource and labour shortage

Palm oil is the largest source of edible oil before soybean oil in the world, which contributes 31.8 % of the total world's production of oils and fats (Basiron, 2007). Before 2007, Malaysia was the largest producer of palm oil, contributed 50.9% of the total production, while Indonesia produced 32.3 %. Malaysia was also the world's largest exporter of palm oil, with 57.3-61.1 % of the world's total exports in 1999-2007. Since 2005, oil palm planted area in Malaysia was 4.05 million hectares and was not expanded due to limited land resources. Although Malaysia has 69 % land area under forest, the government policy restricted the opening up of new land for oil palm plantation. In consequence, Indonesia has become the world's largest producer in 2008, contributing to approximately 50 % of world palm oil production. Nevertheless, the demands of palm oil will be double in the year 2020 exceeding 57 million tonnes (62 %) of the total world's production of oils and fats. This is due to the increasing world population which is expected to be seven billions in the year 2020. Thus, larger oil palm plantation and more workers are needed, and this will be expected to be a major problem by the competition with population area and modernization of the jobs than agriculture sectors.

I.1.2 Wide applications of palm oil

The relatively high level of palmitate (~44 %) provides a degree of stability to the palm oil that makes it suitable for high-temperature frying applications (Sambanthamurthi *et al*., 2000). Palm oil was also proven to be nutritious. The approximately 40 % oleate and 10 % linoleate of palm oil has been shown to lower total serum cholesterol, responsible for the movement of cholesterol within the bloodstream, by lowering the level of the low-density lipoprotein cholesterol (LDL) (Sundram *et al*., 2003). Furthermore, the high level of the desirable high-density lipoprotein cholesterol (HDL) associated with the removal of cholesterol from the bloodstream, has been proven to protect against heart diseases (Sundram, 1997). Thus, about 80 % of palm oil are used for food industries such as cooking oil, shortenings, margarines, ice creams and cookies. Another 20 % are used as feedstock for a number of non-food applications such as oleochemicals and, increasingly, biodiesel (Salmiah,

2000). The invention of new applications in oleochemicals making the demand for palm oil is increasing in last decades. In last few years, the demand of palm oil to be used in biodiesel is significantly increasing particularly from Europe countries. Since the price of fuel based petroleum is increasing in every year, Europe countries also seek to use biodiesel to fulfil its obligations under the Kyoto agreement (Murphy, 2007).

I.1.3 Problem with conventional breeding

All oil palm plantations in Malaysia are cultivated with the eight generation of *Elaeis guineensis*, tenera oil palm. The most common types of *E. guineensis* are dura, tenera and pisifera, which are defined according to endocarp or shell thickness and mesocarp content. The dura fruit has a 2-8 mm thick endocarp and medium mesocarp content of 35-55 % of fruit weight. Meanwhile, the tenera fruit has 0.5-3 mm thick endocarp and high mesocarp content of 60-95 %, and the pisifera fruit has no endocarp and approximately 95 % mesocarp. The tenera is hybrid oil palm derived from crosses between dura and pisifera using conventional breeding approach. The breeding of first tenera oil palm requires almost 12 years and 40 years for eight generations. This was achieved by using large planting area due to open pollinating behaviour of oil palm. Therefore, the production of new traits or varieties of oil palm by conventional breeding is significantly slow. Nevertheless, several traits such as dwarf palms and plants with high vitamin E and oleic acid content have been produced. However, the oil palm has a major problem with incomplete dominance inheritance. For example, the tenera fruits showed all three fruit forms, dura: tenera: pisifera, in a ratio of 1:2:1, indicates the incomplete dominance dura over pisifera. This means only 50 % of the fruits maintained the property of tenera. This problem makes the propagation of oil palm through seeds germination is unsatisfactory.

As above, limited land resources for oil palm plantation, labour shortage, wide applications in oleochemical, biodiesel and problems with conventional breeding and seeds propagation, it is important to find the strategies to solve the mentioned aspects. Two of the strategies are already on the way as the propagation oil palm through tissue culture and oil palm genetic engineering.

I.2 Oil palm tissue culture

Since oil palm is a single growing apex and basal shoots cannot be produced, the propagation of oil palm through vegetative tissue culture is impossible. Thus, the oil palm tissue culture depends exclusively by somatic embryogenesis approach. By using somatic embryogenesis, since 1974, significant progress has been made in the propagation of oil palm through tissue culture (Jones, 1974; Paranjothy and Othman, 1982; Teixeira *et al*., 1993; Teixeira *et al*., 1994; Rival *et al*., 1998). However, the detail information such as optimum media, hormones and the technique of cultures is very limited since most of the works were conducted by the private oil palm companies. At Malaysian Palm Oil Board (MPOB), generally, conventional oil palm tissue culture was based on solid media culture (Figure I.1). However, the long process (52-55 months) of solid media culture produced high percentage (~10 %) of the abnormality of oil palm (Rohani *et al*., 2003) which was mainly due to somaclonal variation. Tissue culturists of MPOB have made extensive improvements, particularly for oil palm suspension cultures which the process has been reduced as a minimum as 35 months (Personal communication: Dr Ahmad Tarmizi, MPOB). Thus, oil palm suspension cultures became a valuable starting material for oil palm genetic engineering to accelerate the transformation process.

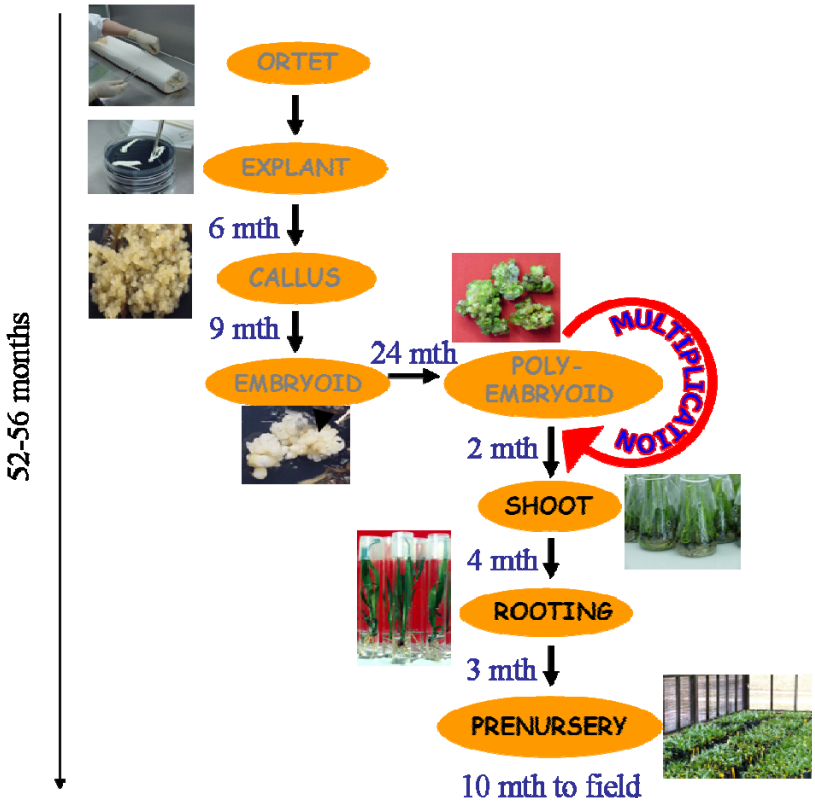

Figure I.1: Propagation of oil palm seedlings through tissue culture.

I.3 Oil palm genetic engineering

The oil palm genetic engineering programme has been initiated at MPOB, then Palm Oil Research Institute of Malaysia (PORIM) in early 1990s (Cheah, 1994). The main objective of this programme is to produce transgenic oil palm with high oleic oil content (Cheah *et al*., 1995). Modification of oil quality such as increasing stearic acid, value-added oil such as synthesizing of palmitoleic acid and ricinoleic acid, and novel products such a biodegradable plastics have also been targeted. It was postulated for oil palm; up to 80 % reduction in time could be achieved for those target products through the combination of genetic engineering and tissue culture (Majid and Parveez, 2007). In addition, since the oil palm is a perennial crop, those products will be continuing produce at least for 30 years, making it an ideal candidate for plant genetic engineering.

At present, biolistic and *Agrobacterium*-mediated transformations are being routinely used to introduce useful genes into oil palm, and the biolistic method has been initially used in oil palm transformation. The factors influenced the biolistic for oil palm transformation such as the appropriate selectable marker (Parveez *et al*., 1996), the choice of promoters (Chowdhury *et al*., 1997), optimal physical (Parveez *et al*., 1997) and biological parameters (Parveez *et al*., 1998) have been identified. Following these factors, the protocol for the production of basta resistance transgenic oil palm was successful developed (Parveez and Christou, 1998). Since this success, thousands of embryogenic calli have been bombarded with various genes such as genes involved in the fatty acid biosynthesis pathway to increase oleic acid and stearic acid (Parveez *et al*., 2000; Ravigadevi *et al*., 2002; Parveez, 2003; Yunus and Kadir, 2008), polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHBV) genes for the production of biodegradable plastics (Parveez *et al*., 2008; Yunus *et al*., 2008; Masani *et al*., 2009). However, biolistic often cause the integration of multiple copies of transgene into the genome of transgenic plants (Stanton, 1998). On the other hand, *Agrobacterium*-mediated transformation has been proven to introduce either single or low copies of transgenes, which was demonstrated from several studies with e.g. rice (Raineri *et al*., 1990) and maize (Gould *et al*., 1991). However, *Agrobacterium*-mediated oil palm transformation still hampers with several limitations such as low transformation frequency since oil palm is not a natural host for *Agrobacterium* like dicotyledon plants. Nevertheless, several studies to improve this method such as the uses of immature embryos (IE) (Ruslan *et al*., 2005) and immature zygotic embryos (IZE) (Bhore and Shah, 2012) as target tissue and the parameters affecting *Agrobacterium*-mediated transformation of oil palm embryogenic calli (Izawati *et al*., 2009;

Yenchon and Te-chato, 2012). A few useful genes such as *Bacillus thuringiensis* insecticidal protein gene (Bt) (Lee *et al*., 2006), Cowpea trypsin inhibitor gene (CpTI) (Ismail *et al*., 2010) to address problems related to insect pests and PHB gene (Ariffin *et al*., 2011), have also been transferred by *Agrobacterium*-mediated oil palm transformation.

Although significant progress was made from both transformation methods, so far, the stability of transgenic oil palms obtained by both methods never been demonstrated particularly the integration and copy numbers of transgenes. Furthermore, those studies have shown that the time required to generate transgenic oil palm was almost 3-5 years, thus, it is expected that the frequency of escape or chimeric plant is high during the selection process. Longer selection process for plant developed from callusing and somatic embryogenesis tends to produce chimeric transgenic plants more frequently. It was suggested that both methods still require optimization of DNA delivery and selection of transformants to overcome the problems associated with escapes.

To overcome problems associated with biolistics and *Agrobacterium*-mediated transformation, an alternative transformation method is urgently required and the transformation of oil palm protoplasts using DNA microinjection is promising approach. However, plant regeneration from oil palm protoplasts is still a challenge and only some few reports are available within the scientific literature describing the regeneration of microcalli from protoplasts.

I.4 History of oil palm protoplasts

The isolation of oil palm protoplasts was first reported by Bass and Huge (1984). The 4-14 days after subculture of suspension culture were digested with enzyme solution consisted of 1/2 strength MS media, 10 % (w/v) driselase and 0.3 M glucose. The yield up to 10^4 protoplasts/ml with viability of >90 % were obtained from 4-14 days after subculture. The protoplasts were cultured either in thin layer liquid media or nurse culture media consisted of 1/2 strength MS supplemented with l g/1 casein hydrolysate, 25 g/1 sucrose and either 2.0 mg/1 2,4-D or 2.5 mg/1 NAA. The protoplasts were developed colonies (7-10 cells) in a month at frequency of 1 % from nurse culture but no further results had been reported.

Three years later, Sambanthamurthi *et al.* (1987) reported the isolation of protoplasts from oil palm polyembryogenic culture with the purpose to determine the fatty acid composition in oil

palm protoplasts. Later, Sambanthamurthi *et al*. (1996) investigated the protoplasts isolation from various tissues, polyembryogenic cultures, vegetative apices of clonal ramets, seed embryos, embryonic axes of germinating seeds, and young inflorescences. Those tissues were plasmolysed by incubation for 30 minutes in A medium, which the A medium was prepared based on Gamborg *et al*. (1968). The plasmolysed tissues were then incubated in various combinations of 2 % (v/v) celluclast, 1 % (v/v) pectinex, 0.25 % (w/v) pectolyase Y23, 1 % (w/v) macerozyme R10, 0.5 % (w/v) hemicellulase and 0.01 % (w/v) trypsin inhibitor, in which sucrose was used as osmostic stabilizer. Among the tissues and enzyme combinations tested, the highest protoplasts yield of 1.5 x 10^6 per g fwt and viability of 95 % was obtained from polyembryogenic cultures digested with enzyme solution consisted of celluclast, pectinex, pectolyase Y23, hemicellulase and trypsin inhibitor. Four types of liquid media, A media, MS media, KM media (Kao and Michayluk, 1975) and WPM media (Russell and McCown, 1986), were used for protoplast culture and subsequently, A media was found as the best media resulted in the formation of microcolonies within 3-4 weeks at frequency of <0.1 %. However, no further growth of microcolonies was obtained. The significant finding from their works was the addition of glutathione and catalase in the media was essential for maintaining the viability of the protoplasts. They also found the protoplasts synthesised palmitoleic acid up to 27 % compared to <0.1 % in oil palm tissue.

1.5 Advantages of DNA microinjection for transformation of oil palm protoplasts

Several advantages of DNA microinjection to be applied for transformation of oil palm protoplasts are as follows.

Small amount of tissue material: Only <0.5 g of oil palm callus is used for protoplast isolation and this will yield thousands of protoplasts subsequently the injection can be performed in the massive of numbers. Thus, thousands of transformant can be obtained from single transformation experiment. The biolistic and *Agrobacterium*-mediated transformation of oil palm requires at least 0.5 g target tissue for each transformation. The preparation of explants is also labour process and time consuming.

Unlimited type of DNA: Various useful genes can be combined for one injection particularly genes involved in the fatty acid biosynthesis pathway. This will speed up the transformation process since the combination of those genes in a vector is time consuming. The DNA can be in the form of linear or circular. For linear DNA, the DNA fragment, promoter-gene-

terminator sequence or “clean gene” can be injected into the oil palm protoplast to produce transgenic oil palm free from selectable marker or backbone vector sequence. This will give the freedom to operate since most of the selectable marker genes or vector sequences are protecting by intellect property, which will reflect the commercialization of the transgenic oil palm in the future. The production of transgenic oil palm without the selectable markers is expected to be much faster than transgenic oil palm produced by the selection process. In addition, only 1-2 pl DNA solution is required for injection. Thus, in total, only 0.1-2 ug/l requires per needle for thousands injections.

Specific compartment: The injection can be performed in nucleus or cytoplasm of oil palm protoplast. The open pollinating behaviour of oil palm makes the cytoplasm of protoplasts is preferably a compartment for oil palm genetic engineering. DNA microinjection into protoplast isolated from green tissue of oil palm is an ideal candidate for plastid transformation. Transgenic oil palm produced will be in the maternal inheritance plants, thus minimizes the possibility of leaking transgenes by pollination to environment particularly weeds and normal oil palm (Daniell *et al*., 2002).

Real time transformation: Since the microinjection of DNA is performed under a microscopy, the transformation can be precisely monitored. The viable protoplasts can be selected and injected, and none injected protoplasts can be destroyed instantly. Thus, only transformed protoplasts will be regenerated.

The available of DNA microinjection techniques: The physical properties of protoplast are similar within plant species. Thus, the microinjection techniques from other plants species could be adopted and applied for oil palm protoplasts. This included the use of extra injection needle to hold the protoplasts (Morikawa and Yamada, 1985; Crossway *et al*., 1986), the use of the ring units for immobilization and culture of the microinjected protoplasts (Lawrence and Davies, 1985; Miki *et al*., 1989), the use of polylysine to attach the protoplasts inside glass cover slip (Reich *et al.*, 1986) and the use of alginate layer embedding protoplasts as the platform for DNA microinjection (Schnorf *et al.*, 1991; Kost *et al.*, 1995).

Stable transgenic oil palm could be produced: Transformation and subsequently integration into the genome of oil palm protoplasts by microinjection could lead to the production of stable transgenic lines since those plants were regenerated from a single

transformed cell. Thus, high transformation frequency and single copy of transgene could be obtained. So far, the transformation frequency of 1-1.5 % from biolistic (Parveez, 2000) and 0.7 % from *Agrobacterium*-mediated (Izawati *et al*., 2009) of oil palm transformation have been achieved and the copy number of transgene of those transgenic oil palm was never been investigated. Meanwhile, DNA microinjection into protoplasts produced transgenic plants with the transformation frequency of 26 % for alfalfa (Reich *et al.*, 1986), 20 % (Schnorf *et al*., 1991) and 53 % (Kost *et al*., 1995) for tobacco, demonstrated that the possibility high transformation frequency could also be achieved for oil palm. In addition, studies from Schnorf *et al*. (1991) revealed that all transgenic tobacco plants contained low copy numbers of transgene (~1). For oil palm, the transgenic oil palm with low copy or accurately single copy transgene is one of the compulsory criteria for commercialization.

I.6 Objective of the study

The long-term objective of the MPOB-IME collaborative project is to establish marker free transgenic oil palms with high added value, recombinant proteins, for commercial exploitation. Recombinant proteins which will be used as high added value products are: PIPP, a chimeric antibody against human chorionic gonadotropin (hCG). This antibody can be used for pregnancy diagnosis and contraception as well as cancer diagnosis, prognosis and immunotherapy; D12, a human antibody against caries; Human Serum Albumin (HSA) used as plasma extender and for other application in clinical medicine.

These products should be exclusively produced in the leaf, mesocarp and kernel tissue of transgenic oil palm. The constructs for D12 and HSA genes have been constructed by IME scientist. Therefore, the first part of the study will be focused on the construction of PIPP gene under the control of three tissue-specific promoters, leaf-specific promoter (LSP), mesocarp-specific promoter (MSP) and kernel-specific promoter (KSP). Since oil palm transformation is a time-consuming process, the functionality of constructs will be evaluated in tobacco plants.

The main objective of this study is to develop the oil palm transformation method based on DNA microinjection approach by using oil palm protoplasts as target cells. The study will involve the optimization of protoplast isolation, selection of suitable oil palm suspension culture and identification of optimum factors for plant regeneration. After the protocol of plant regeneration from oil palm protoplasts has been developed, the possibility of oil palm protoplasts to be used for DNA microinjection will be evaluated by PEG-mediated transformation. Finally, the optimal parameters for DNA microinjection will be verified and optimized. The steps taken throughout the study is summarized in Figure I.3.

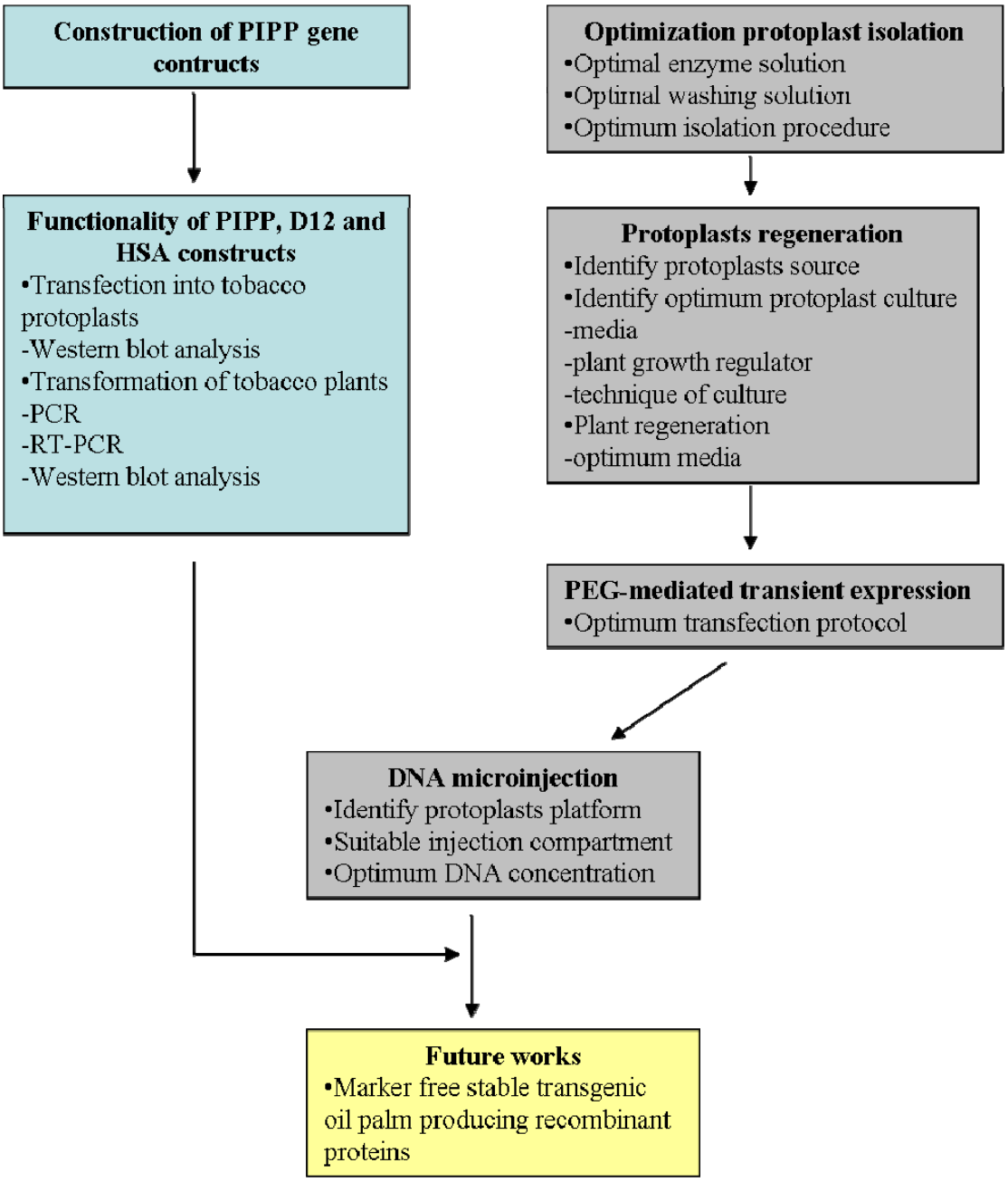

Figure I.2: Steps taken to achieve the main objective of the study.

II MATERIAL AND METHODS

II.2 Material

II.2.1 Oligonucleotides

Oligonucleotides used for PCR amplifications and sequence analyses are listed below. All oligonucleotides were synthesised by Metabion International AG (Germany).

Primers used for PCR amplification of target genes

Primer name	Nucleotide sequence (5' to 3')	Gene (bp)
PIPPlight fw EcoRI	AGAGAATTCACACAATCAGATTTA	PIPP-light chain (824)
PIPPlight rev XhoI	TCTCTCGAGCTAGAGCTCATCTTTCTC	
PIPPheavy fw EcoRI	AGAGAATTCACACAATCAGATTTATAGAG	PIPP-heavy chain (1493)
PIPPheavy rev XhoI	TCTCTCGAGCTACCCCTCGACTTTAC	
35ST XhoI fw	AGACTCGAGGTCCGCAAAAATCACCA	35S terminator (234)
35ST KpnEcoRVrev	AGAGGTACCGATATCGTCACTGGATTTTGGTTTTA	

Primers used for DNA sequencing

Primer name	Nucleotide sequence (5' to 3')
PIPP light fw Mitte raus	TCTGGATTTATAGCACATC
PIPP light rev Mitte raus	CCTCTCTGGGATAGAAG
PIPP heavy fw raus	CCACGAAGACCCTGAG
PIPP heavy rev vorn raus	GTCCTGAGGACTGTAG
PIPP heavy fw Mitte raus	CTCAGCCTCCACTAAG
M13 fw	GTAAAACGACGGCCAG
M13 rev	CAGGAAACAGCTATGAC

Primers used for PCR analysis of transgenic tobacco plants

Primer name	Nucleotide sequence (5' to 3')	Gene (bp)
LSP 550 fw	GCAACTCAAAACTCAAAT	LSP-GFP_{ER} (497)
GFPbw	AAGTAGTGACAAGTGTTGG	
MSP fw 410	GGTGTAGGATTTGTG	MSP-GFP_{ER} (691)
GFPbw	AAGTAGTGACAAGTGTTGG	
KPE SmaI fw	AGACCCGGGTCAAATTATCAAAATATC	KSP (402)
KPE rev raus	CTGACCTGTGACTTCTC	
LSP 550 fw	GCAACTCAAAACTCAAAT	LSP-hyg (610)
Hy rev vorn raus	GAAAGCACGAGATTCTTC	

D12 light fw EcoRI D12 light rev XhoI	AGAGAATTCCGCTGAATTCACAACACA AGACTCGAGTTTAACACTCTC	D12-light chain (767)
D12 heavy fw BspHI D12 heavy rev XbaI	AGATCATGAGCTGAATTCACAACACAA AGATCTAGACGACCTAAAGTTCATCCT	D12-heavy chain (1546)
HSA fw HSA rev raus	ATGGACTTTCAAGTGCAG AGCAGCTTGGCAACATTCT	HSA (582)

Primers used for RT-PCR

Primer name	Nucleotide sequence (5' to 3')	Gene (bp)
GAPDH fw GAPDH rev	AGCTCAAGGTTAAGGATGAC TGGCCAAGGGAGCAAGGCAA	GAPDH (gDNA: 1100; cDNA: 300)
HSA-Fw HSA-Bw	AAAGCATGGGCAGTAGCTCGCC TGCAGCGGCACAGCACTTCT	HSA (459)
D12Lc-Fw D12Lc-Bw	TTCTTGCTCAGCGGCACTGC AGGCACACAACAGAGGCAGCT	D12-light chain (443)
D12Hc-Fw D12Hc-Bw	ACCTGGAGCGAAAGCGGACA TGGGCAGCAGTGCAGGTGAA	D12-heavy chain (467)
PHC01-F PHC02-R	GGCCTTGAGTGGATTGGAGA TTGGTGTTGCTGGGCTTG	PIPP-heavy chain (509)
PLC01-F PLC02-R	GGTACCAGCAGAAGCCAGGA AACCTCCACCTCCGTCGACA	PIPP-light chain (558)
EgAct2-Fw EgAct2-Bw	TGCCTGATGGGCAGGTCATCAC TTCCGGTGCACGATTGCAGGAC	actin (gDNA: 600; cDNA: 400)
LS01-F LS02-R	AGAGCACCTACCCAACAGCA CGAACTTGACGCCATTGC	oil palm LHCB (451)
MS01-F MS02-R	ATGATGAGCATGTCTTGGAACC TCCTGCAAATTCCAAGCC	oil palm MT3-A (565)
KS01-F KS02-R	AATGCCCTCGAGCCGACAA CCAGCTCCATGTTAACGCCA	Oil palm glutelin (580)

II.2.2 Primary antibodies and secondary antibodies

The antibodies used for western blot analysis are listed below.

Target protein	Primary antibody	Secondary antibody
PIPP	Rabbit Anti-Human IgG (H+L) (Thermo scientific)	Stabilized Goat anti-rabbit IgG (H+L) (Thermo scientific)
D12	Rabbit Anti-Human IgG (H+L) (Thermo scientific)	Stabilized Goat anti-rabbit IgG (H+L) (Thermo scientific)
HSA	Monoclonal anti-human serum albumin clone HSA-11 (Sigma)	Stabilized Goat anti-mouse IgG (Thermo scientific)

II.2.3 Plasmid DNA

The plasmids used for the DNA manipulation are listed below.

Plasmids	Description	Source
pBluescriptII KS^+	commercial cloning vector	Stratagene
pCRII-TOPO	commercial cloning vector	Invitrogen
cPIPP	heavy and light chain of PIPP gene	IME
LSP-HSA MSP-HSA KSP-HSA	HSA gene driven by oil palm leaf specific (LSP), mesocarp specific (MSP) and kernel specific (KSP) promoters	IME
LSP-hyg-LSP-HSA LSP-hyg-MSP-HSA LSP-hyg-KSP-HSA	HSA gene driven by LSP, MSP and KSP promoters and linked to the hygromycin (hyg) gene driven by LSP promoter	IME
LSP-hyg-LSP-D_{12}HLc	Each heavy and light of D12 genes driven by LSP promoter and linked to the hyg gene driven by LSP promoter	IME
pBin19	Ti vector contain nptII gene driven by Nos promoter	IME
Cambia0380	Ti vector	Cambia
pBS1029	ER targeting GFP gene and 35ST terminator gene	IME

II.2.4 *Escherichia coli* and *Agrobacterium tumefaciens* strains

Strain	Relevant genotype and/ or phenotype	Reference / source
E. coli DH10B	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z_M15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ-*rps*L *nup*G	Life Technologies, Germany)
E. coli NEB10-β	*araD*139 Δ(*ara-leu*)7697 *fhuA lacX74 galK* (Φ80 Δ(*lacZ*)*M15*) *mcrA galU recA1 endA1 nupG rpsL* Δ(*mrr-hsdRMS-mcrBC*)	New England Biolabs, USA
A. tumefaciens LBA4404	pTiA6, Rif^R	Hoekama *et al*. (1983)

II.2.5 Plant materials

Nicotiana tabacum var. SR1 plants were used for protoplast transient expression and stable transformation.

Oil palm suspension cultures (clone R160) were used for protoplasts regeneration, PEG-mediated transformation and DNA microinjection.

II.2.6 Solutions, buffers and media for tobacco work

Solutions, buffer and media used throughout the studies using the tobacco plants are list below.

Name	Component	Concentration
K3 medium	MS salt + vitamins Sucrose Xylose	4.4 g/l 140 g/l 0.25 g/l pH 5.6
W5 medium	NaCl $CaCl_2.2H_20$ KCl Glucose	9 g/l 18.4 g/l 0.37 g/l 0.991 g/l pH 5.6
MaMg solution	Mannitol $MgCl_2.6H_2O$ MES	84.23 g/l 3.05 g/l 1 g/l pH 6
PEG solution	$Ca(NO_3)_2.4H_2O$ Mannitol PEG 4000	23.6 g/l 72.9 g/l 400 g/l pH 7
0.5 mM NaPP Buffer	0.2 M Na_2HPO_4 0.2 M NaH_2PO_4	152.2 ml/l 97.5 ml/l
12 % Resolving gel	40 % Acrylamide/0.8 % bisacrylamide 1.88 M Tris-HCl, pH 8.8 0.5 % SDS ddH_2O TEMED 10% APS	2772 µl 1980 µl 1980 µl 3168 µl 8.25 µl 50 µl
5 % Stacking gel	40 % Acrylamide/0.8 % bisacrylamide 0.625 M Tris-HCl, pH 6.8 0.5 % SDS ddH_2O TEMED 10 % APS	756 µl 1200 µl 1200 µl 2855 µl 6 µl 30 µl

5x SDS reducing buffer	ddH_2O	6.8 ml
	0.5 M Tris-HCl, pH 6.8	2 ml
	Glycerol	3.2 ml
	20 % SDS	1.6 ml
	β-Mercaptoethanol	0.8 ml
	BFB	0.016 g
10x Electrode running buffer	Tris base	30.3 g/l
	Glycine	144 g/l
	SDS	10 g/l
Coomassie staining solution	Coomassie brillant blue G-250	0.25 % (w/v)
	Methanol	50 % (v/v)
	Glacial acetic acid	9 % (v/v)
Coomassie destaining solution	Methanol	10 % (v/v)
	Glacial acetic acid	10 % (v/v)
10x PBS buffer	NaCl	81.8 g/l
	KCl	2 g/l
	KH_2PO_4	2.45 g/l
	$Na_2HPO_4.H_20$	13.8 g/l
		pH 7.2
10x Western blot buffer	Glycine	144.1 g/l
	Tris base	30.2 g/l
		pH 8.3
PBS-T buffer	10x PBS buffer	1x
	Tween-20	0.05 %
		pH 7.2
YEB medium	Beef extract	0.5 % (w/v)
	Yeast extract	0.1 % (w/v)
	Peptone	0.5 % (w/v)
	Sucrose	0.5 % (w/v)
	$MgSO_4$	2 mM
		pH 7
YEB agar	YEB medium	1.8 % (w/v)
	Agar	pH 7
MSI medium	MS salt + vitamins	4.4 g/l
	Glucose	30 g/l
		pH 5.8
MSII agar	MS salt + vitamins	4.4 g/l
	Glucose	30 g/l
	Agar	7 g/l
	BAP	0.5 mg/l
	NAA	0.1 mg/l
		pH 5.8

MSIII agar	MS salt + vitamins Sucrose Agar	4.4 g/l 20 g/l 7 g/l pH 5.8
Edwards' extraction buffer	Tris-HCl, pH 7.5 NaCl EDTA SDS	200 mM 250 mM 25 mM 0.5 % (w/v)
TE buffer	Tris-HCl EDTA	10 mM 1 mM pH 8
QB buffer	KPO_4, pH 7.8 EDTA Triton X-100 Glycerol DTT	100 mM 1 mM 1 % (v/v) 10 % (v/v) 1 mM

II.2.7 Solutions, buffers and media for oil palm work

Table 1I.1: Composition of enzyme solutions

Components (w/v %)	Enz Sol I	Enz Sol II	Enz Sol III	Enz Sol IV	Enz Sol V	Enz Sol VI
Celluclast	2	2	2	2	2	2
Pextinex 3XL	1	1	1	1	1	1
Pectolyase Y23	0.25	0.25	0.25		0.25	0.1
Cellulase onuzuka R10		0.5	0.5	0.5		0.5
Hemicellulase	0.5			0.5		
Macerozyme					0.5	
Sucrose	8					
Mannitol		7.28	3.6	3.6	3.6	3.6
KCl			1	1	1	3
$CaCl_2.2H_20$			0.5	0.5	0.5	0.5

Table II.2: Composition of washing solutions

Components (w/v %)	Wash Sol I	Wash Sol II	Wash Sol III	Wash Sol IV
Mannitol	7.28	3.6		3.6
Glucose			0.01	
KCl		1.5	0.04	3
$CaCl_2.2H_20$		0.98	0.8	0.5
NaCl			0.9	

Table II.3: Composition of PGRs used

PGRs combination	Auxin (µM)				Cytokinin (µM)			
No.	NAA	2,4-D	IAA	IBA	Zea	GA3	BA	2iP
1	1	11	1	11	1	11	1	11
2	2	10	2	10	2	10	2	10
3	3	9	3	9	3	9	3	9
4	4	8	4	8	4	8	4	8
5	5	7	5	7	5	7	5	7
6	6	6	6	6	6	6	6	6
7	7	5	7	5	7	5	7	5
8	8	4	8	4	8	4	8	4
9	9	3	9	3	9	3	9	3
10	10	2	10	2	10	2	10	2
11	11	1	11	1	11	1	11	1
12	10		10	2	10	2	10	2
13	10	2		2	10	2	10	2
14	10	2	10		10	2	10	2
15	10	2	10	2		2	10	2
16	10	2	10	2	10		10	2
17	10	2	10	2	10	2		2
18	10	2	10	2	10	2	10	
19	10	2		2		2		2

II.2 Methods

II.2.1 DNA manipulation

II.2.1.1 PCR amplification

PCR amplification was performed to amplify the genes of interest and to identify the positive colonies of bacterial or agrobacteria clones. The accuprime *pfx* DNA polymerase (Invitrogen) with proof reading activity was used for amplification of gene of interest and the Mango*Taq* DNA polymerase (Bioline) for identification of the PCR colony. The reaction mixtures and programmes of the PCR amplification were performed according to the manufacturer's instructions.

II.2.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed according to Sambrook and Russel (2001). The PCR products or DNA fragments were separated by electrophoresis through a 1 % (w/v) agarose gel. The gel was submerged in a sufficient amount of 1x TAE buffer prior to loading of the DNA samples. The DNA samples to be loaded were mixed with 1x loading buffer. The gel electrophoresis was run with the standard size marker (1 kb DNA ladder; Invitrogen: Figure II.1) at 100 V for 30-60 minutes. After electrophoresis, the gel was visualized on a UV transilluminator for further analyses.

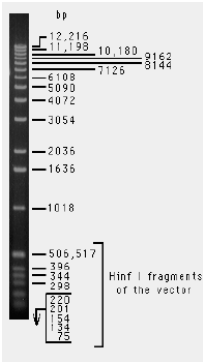

Figure II.1: 1 kb DNA ladder (Invitrogen)

II.2.1.3 Isolation of DNA fragments from agarose gel

The DNA fragments that were required for DNA manipulation was isolated by the means of agarose gel electrophoresis. The DNA fragments were excised using a clean razor blade and

isolated using the NucleoSpin® Gel Extraction according to the manufacturer's instructions (Macherey-Nagel, Germany).

II.2.1.4 Restriction enzyme digestion

All of the restriction enzyme digestions were carried out under the conditions recommended by the supplier of the specific enzyme. Restriction enzyme digestions were performed to analyze plasmid DNA and to isolate DNA fragments for DNA manipulation. Typically, the digestions were carried out in a 20 µl reaction volume using 1 unit of the enzyme for 1 µg of DNA, where a unit of enzyme is defined as the amount of enzyme required to completely digest 1 µg of DNA at the recommended temperature for 1 hour (Sambrook and Russel, 2001).

II.2.1.5 Formation of DNA with blunt ends

To allow ligation between the DNA fragments or vectors which had been digested with restriction enzymes which created sticky ends into the cohesive ends in some of the DNA manipulation, the sticky ends were first rendered blunt. The cohesive ends were filled in a reaction volume using 0.5 units of the Accuprime *pfx* DNA polymerase (Invitrogen), 1 mM dNTPs mixture, 1x *pfx* amplification buffer, 1 mM $MgSO_4$ followed by incubation at 72°C for 30 minutes and then the reaction was purified using the PCR Purification Kit (Macherey-Nagel, Germany).

II.2.1.6 Dephosphorylation

To prevent self ligation of the vector DNA, the 5' phosphate group was removed from the vector DNA by treatment with 1 unit of Calf Intestinal Phosphatase (CIP) (New England Biolabs) in a 30 µl reaction volume. The mixture was incubated at 37°C for 1 hour then the reaction was terminated and purified using the PCR Purification Kit (Macherey-Nagel, Germany).

II.2.1.7 Ligation

The ligations were performed in a 20 µl reaction using a T4 DNA ligase according to the manufacturer's instructions (Promega). The DNA insert was used in an equal or up to 3 fold molar concentration over DNA vector. For the ligation of DNA with sticky ends, 1 unit of T4 DNA ligase was used and the ligation mixture was incubated at room temperature for 1-3

hours. For the DNA with blunt ends, the reaction was supplemented with 2 units of T4 DNA ligase and incubated overnight at 16°C. One-3 µl of the ligation mixture was used directly for transformation of *E. coli* competent cells.

II.2.1.8 Transformation of *E. coli* by electroporation

Electrocompetent *E. coli* cells were prepared for DNA transformation as described by Dower *et al.* (1988). A 50 µl aliquot of electrocompetent *E. coli* cells, DH10B or NEB10-β, was thawed on ice and the cells was mixed with DNA solution. The cells was transferred into a pre-chilled electroporation cuvette (0.1 cm) and a Gene Pulser (BIORAD) was set to 25 µFD capacitance 1.5 kV charge and the Pulse Controller was set to 200 Ω resistance. After application of the pulse for 8-12 ms, the cells were diluted with 800 µl LB medium and incubated at 37°C for 45 minutes. The cells was pelleted by centrifugation at 4000 rpm for 5 minutes and spread onto LB agar containing appropriate antibiotics and incubated overnight at 37°C.

II.2.1.9 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated using a NucleoSpin Plasmid QuickPure Kit or midi scale Plasmid DNA Purification Kit (NucleoBond PC100) according to the manufacturer's instructions (MACHEREY-NAGEL, Germany). The concentration and purity of plasmid DNA was determined by using spectrophotometric method and stored at -20°C.

II.2.1.10 Determination of DNA concentration

The concentration of the DNA samples was determined by using spectrophotometric method. An absorbance unit of 1 at 260 nm represents approximately 50 µg/ml of double stranded DNA (Sambrook and Russel, 2001). The purity of the DNA samples was determined by comparing the measured absorbance at 280 nm and 260 nm which a ratio of approximately 1.8-2 for pure DNA, while values below 1.8 indicates samples are contaminated with proteins.

II.2.2 Transient transformation of tobacco protoplasts

II.2.2.1 Isolation of tobacco protoplasts

The in vitro young tobacco leaves were cut into small pieces and transferred into an 50 ml flask containing filter-sterilized K3 medium supplemented with 0.7 % (w/v) cellulase onuzuka R10 (Duchefa) and 0.5 % (w/v) macerozym (Duchefa) and incubated in the dark at 26°C for overnight. After incubation, the digested leaves were resuspended by shaking at 100 rpm for 30 minutes and then filtered through a sterilized steel sieve (250 μm) by collecting in 50 ml centrifuge tube. The tube was centrifuged at 100 xg for 5 minutes at 22ºC and the floating protoplasts was transferred into new 50 ml centrifuge tube, and then centrifugation was repeated. The protoplast pellet was resuspended by inverting the tube after adding 40 ml of W5 medium, and then centrifuged at 100 xg for 2 minutes at 22ºC. After repeating the washing step 2 times, the supernatant was removed completely and the protoplast pellet was resuspended with 5 ml filter-sterilized MaMg solution and stored at room temperature or 4ºC.

II.2.2.2 Polyethylene glycol (PEG) transfection of tobacco protoplasts

The PEG transfection of tobacco protoplasts was performed according to the method described by Negritiu *et al.* (1987) with some modifications. A 330 μl aliquot of the protoplasts suspension was transferred into 10 ml culture tube and incubated at room temperature for 10 minutes. Fifteen μl (10 μg) of plasmid DNA was added slowly to the protoplasts suspension, and mixed carefully by tapping the tube and incubated in the dark at 26°C. After incubation for 10 minutes, 330 ul PEG solution was added drop by drop to the DNA-protoplasts suspension and incubated for another 25 minutes, then 4 ml K3 medium was added drop by drop and incubated in the dark at 26ºC for overnight.

II.2.2.3 Protein extraction of tobacco protoplasts

The transfected protoplasts was transferred into 1.5 ml eppendorf tube and centrifuged at 11000 rpm for 1 minute at 4 ºC. The supernatant was discarded and the protoplast pellet was resuspended by pipetting with 1 ml tip after adding 50 μl of 50 mM NaPP buffer, and then centrifuged again. The supernatant containing extracted protein was transferred into new eppendorf tube. The concentration of protein was measured using Bradford solution and stored in -20°C.

II.2.2.4 Determination of proteins concentration

Proteins concentration was determined according to the method described by Bradford (1976). Five µl of protein samples was mixed with 1 ml diluted (1:5) Roti® Quant Bradford-Reagent. After 10 minutes incubation at room temperature, the absorbance at 595 nm was measured against a blank reagent prepared from 5 µl of the QB buffer and 1 ml diluted (1:5) Roti® Quant Bradford-Reagent. The protein concentration was obtained by calibration of Bovine serum albumin which was used as a standard.

II.2.2.5 SDS-Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was used for the separation of protein samples under denaturing conditions according to Laemmli (1970) by using 12 % Resolving gel and 5 % Stacking gel. SDS-PAGE was performed using the Mini-PROTEN 3 Cell apparatus according to the manufacturer's instructions (BIORAD). Fifty mg protein sample was mixed with 5x SDS reducing buffer and denatured by boiling at 95°C for 5 minutes, and then immediately chilled on ice. The denatured protein sample was loaded into gel wells submerged in 1x Electrode running buffer and the electrophoresis was performed at constant 30 mA for 80 minutes. The gel was placed in coomassie staining solution and stained until the protein band was observed. Nonspecific background was removed using coomassie destaining solution.

II.2.2.6 Western blot analysis

The separated proteins in SDS-PAGE were blotted onto Hybond-ECL-nitrocellulose membrane (0.45 µm) by using the Mini Trans-Blot® Electrophoretic Transfer Cell apparatus according to the manufacturer's instructions (BIORAD). Briefly, SDS-PAGE gel, nitrocellulose membrane, whatman filter paper and fiber pad were soaked in 1x PBS buffer for few minutes and assembled as sandwich in the gel holder cassette. The sandwich cassette was transferred to the buffer tank containing 1x western blot buffer and placed in an icebox. Protein blotting was performed at 200 mA for 60 minutes. The sandwich cassette was disassembled and the membrane was carefully removed and rinsed 2 times with 1x PBS buffer. The membrane was blocked with 1x PBS buffer containing 5 % (w/v) skimmed milk powder at 4°C for overnight. The blocking solution was replaced with PBS-T buffer containing 1 % (w/v) skimmed milk powder with primary antibody and incubated at room temperature for 90 minutes. The membrane was washed 2 times with PBS-T buffer for 30 minutes each and then incubated in PBS-T buffer containing 1 % (w/v) skimmed milk powder with secondary antibody at room temperature for 90 minutes. Then the membrane

was washed 3 times with PBS-T buffer for 30 minutes each. The detection of target proteins was performed using SuperSignal West Dura Extended Duration Substrate (Thermo scientific) according to the manufacturer's instructions.

II.2.3 Stable transformation of tobacco plants

II.2.3.1 Transformation of *Agrobacterium tumefaciens* competent cells

A 50 µl aliquot of A. tumefaciens LBA4404 cells was thawed on ice and then 100 ng of plasmid DNA was added to the thawed cells. The cells was kept on ice for 2 minutes while a Gene Pulser (Biorad) was set to 25 µFD capacitance 2.5 kV charge and the Pulse Controller was set to 200 Ω resistance. The cells was transferred to a pre-chilled electroporation cuvette (0.2 cm) and slid between the contacts of the reaction chamber allowing an electrical pulse (8-12 ms) to be initiated. The cuvette was immediately removed from the chamber and 1 ml of YEB medium was added. The cells was transferred to a centrifuge tube and incubated at 28°C for 3 hours. A 50 µl aliquot of the cells was spread on YEB agar containing 100 µg/ml rifampicin (Rif) and 50 µg/ml kanamycin (Km) (YEB-Rif-Km). The plates were incubated at 28°C for 2 days.

II.2.3.2 Leaf-disc transformation of *N. tabacum* cv. Petite Havana SR1

The colonies of recombinant *A. tumefaciens* were verified for the presence of plasmid DNA by PCR amplification. Positive colonies were inoculated into 5 ml of YEB-Rif-Km medium and incubated at 28°C for 2 days with shaking at 150 rpm. Two ml culture cells was transferred to 50 ml Falcon tube containing 40 ml YEB-Rif-Km medium and incubated overnight. The cells was further cultivated until an optical density of 1 at 600 nm was achieved then the cells were pellet by centrifugation at 4000 rpm for 5 minutes. The cells were resuspended in 40 ml 10 mM $MgSO_4$ and recentrifuged, and then resuspended in 40 ml MSI medium as agrobacteria suspension. The in vitro young tobacco leaves were cut into small pieces and immersed in agrobacteria suspension and then incubated in the dark for 2 hours. The leaf pieces were transferred upside down onto MSI agar and incubated at 25°C in the dark for 2 days. Then, the leaf pieces were washed 3 times in MSI medium containing 500 mg/l claforan. The leaf pieces were blotted dry on sterile whatman paper and were cultured upside down onto MSII agar containing 500 mg/l claforan and 100 mg/l kanamycin or 10 and 50 mg/l hygromycin and incubated at 25°C under 16 hours photoperiod. The leaf

pieces were transferred every 2 weeks onto fresh MSII agar until callus and shoots developed. The shoots were transferred onto MSIII agar containing 500 mg/l claforan and 100 mg/l kanamycin or 10 and 50 mg/l hygromycin and incubated at 25°C under 16 hours photoperiod until plantlets with roots developed and then transferred into soil.

II.2.3.3 Extraction of genomic DNA from tobacco plants

Genomic DNA was extracted from transgenic and wild type tobacco plants by using the SDS alkaline method developed by Edwards *et al.* (1991) with some modifications. The leaf samples were placed in 1.5 ml eppendorf tube and ground together with 400 µl Edwards' extraction buffer. The eppendorf tube was vortexed at high speed and centrifuged at 14000 rpm for 1 minute. Three hundred µl supernatant was transferred into a new eppendorf tube and mixed with 300 µl isopropanol, and then incubated at room temperature for 2 minutes. The DNA was pelleted by centrifugation at 14,000 rpm for 2 minutes. The pellet was then washed with 200 µl 80 % ethanol and dissolved in 100 µl TE buffer. Five µl genomic DNA was used for PCR analysis to detect the transgenes using designed primers as listed (II.2.1).

II.2.3.4 Extraction of total RNA from tobacco plants

The extraction of total RNA from tobacco leaves was performed using a NucleoSpin RNA® Plant Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Approximately 100 mg of tobacco leaves was frozen and ground to a fine powder in liquid nitrogen to be used with the kit.

II.2.3.5 Reverse Transcription-PCR (RT-PCR)

First strand cDNA synthesis was performed using a SuperScript II (Invitrogen, Germany) according to the manufacturer's instructions with some modification. The total RNA samples were digested with DNase enzyme to remove the genomic DNA. One unit of the DNase enzyme (New England Biolabs) was added to 5 µg of the isolated RNA and incubated at 37°C for 10 minutes, and then incubated at 65°C for 5 minutes. Five µl (2 µg) DNase treated RNA was mixed with 1 µl Oligo-dT 18 primer (10 pmol/µl) and 5 µl ddH_2O and incubated at ramp temperatures of 80/ 72/ 50/ 42/ 37/ 20°C for 5 minutes each. Then, the reaction mixture was placed on ice and mixed with 4 µl 5x first-strand buffer, 2 µl DTT and 1 µl dNTPs, and then incubated at 42°C for 2 minutes. One µl Superscript II-RT enzyme was added to the reaction mixture and incubated at 42°C for 50 minutes and 70°C for 15 minutes, and then the reaction mixture was placed on ice. The trace amount of RNA in the reaction mixture was

removed by incubation with 0.5 µl RNaseH enzyme (New England Biolabs) at 37°C for 20 minutes. Two µl of the synthesized cDNA was used as a template for PCR amplification using designed primers as listed (II.2.1).

II.2.3.6 Proteins extraction of tobacco leaves

The extraction of total proteins from tobacco leaves was performed according to the method described by Ni *et al.* (1996). One gram frozen leaves were ground in 2 ml of QB buffer. The ground samples were transferred into 2 ml eppendorf tube followed by top speed centrifugation at 4°C for 15 minutes. The supernatant was transferred in a 1.5 ml eppendorf tube and the centrifugation step was repeated. Then, the supernatant was transferred into a new 1.5 ml eppendorf tube. The concentration of protein in the supernatant was measured using Bradford solution and the isolated protein was stored in -20°C.

II.2.4 Biolistic transformation of oil palm embryogenic calli

The plasmid DNA was coated with gold particles according to manufacturer's manual for biolistics Particle Delivery System, PDS-1000/He (BIORAD). The gold particles were prepared by placing 60 mg of gold particles in 1 ml of 100 % ethanol and vortexed for 3 minutes. The gold particles were centrifuged at 10,000 rpm for 10 seconds followed by the removal of supernatant. The gold particles were washed with 1 ml sterile water, vortexed, centrifuged before supernatant was removed. These procedures were repeated once. The gold particles were then resuspended in 1 ml sterile water and dispensed into 100 µl aliquots and stored at 4°C.

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 minutes followed by centrifugation at 10,000 rpm for 10 seconds. The supernatant was removed as much as possible and washed twice with 500 µl 100 % ethanol, followed by mixing, and centrifugation at 10,000 rpm for 60 seconds. Finally, the DNA coated gold particles were resuspended in 120 µl 100 % ethanol. For each bombardment, about 6 µl of DNA coated gold particles were dispensed in the centre of a macrocarrier and dried under a sterile condition and used immediately for particle bombardment. Embryogenic calli which were placed in the centre of solid medium were used as target tissues. Transformation was carried out using the following parameters: bombardment pressure at 1100 psi; macrocarrier to stopping screen distance at 6 mm; target plates distance at 6 cm; chamber vacuum at 26 inch Hg (Parveez, 1998).

II.2.5 Protoplast isolation from oil palm tissues

II.2.5.1 Plant material

Oil palm embryogenic cells suspension was maintained in 100 ml flask containing 50 ml Y3 liquid medium (Appendix 1) supplemented with 5 µM NAA, 5 µM 2.4-D and 2 µM 2iP. This medium is designated "Y35N5D2iP". The embryogenic cells suspension was collected by filtration with 300 µm nylon mesh and transferred onto ECI agar medium (0.7 % agar) (Appendix 1) supplemented with 5 µM NAA to form embryogenic calli, embryos, polyembryogenic calli and small plantlets.

II.2.5.2 Protoplast isolation and purification

For each experiment, 0.5 g plant material was transferred into 50 ml centrifuge tube containing 15 ml of different filter-sterilized enzyme solutions (Table II.1). The mixture was resuspended in enzyme solution by inverting the centrifuge tube for 6-10 times. The centrifuge tube was incubated in a horizontal position in the dark either by shaking at 60 rpm or static position for 1-24 hours at 24-28ºC. After incubation, the mixture was diluted by 15 ml of filter-sterilized washing solution consisting of different components (Table II.2). The diluted mixture was resuspended by inverting the centrifuge tube for 3-5 times and then filtered through a sterilized double layer miracloth by collecting in 50 ml centrifuge tube. The filtration step was repeated 2-3 times until all the undigested tissues, cell clumps and cell wall debris were removed. The centrifuge tube was centrifuged at 60 xg for 5 minutes at 22ºC and the supernatant was almost removed leaving 5 ml. The protoplast pellet was resuspended by inverting the tube after adding 10 ml of washing solution, and then centrifuged again. After repeating the washing step 3 times, the supernatant was removed completely and the protoplasts pellet was resuspended with 5 ml filter-sterilized Rinse solution consisted of 3 % (w/v) KCl and 3.6 % (w/v) mannitol at pH 5.6.

II.2.5.3 Protoplast yield and viability

The yield and viability of the purified protoplasts were calculated with a Nageotte hematocytometer in 3 replicates for each independent experiment. The following formula was used as $X = Y \times 10^5/Z$ [X is the number of protoplasts per ml, Y is the average quantity of protoplasts in 5 x 1 mm^2, Z is the fresh weight (fwt) of plant material in grams] as the protoplast yield, whereas the viability was calculated as the number of protoplast fluorescing green after stained by 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, AM; Invitrogen) divided by the protoplast yield in percent.

II.2.6 Protoplast isolation from oil palm suspension culture

II.2.6.1 Oil palm suspension culture

Oil palm embryogenic cell suspensions were cultured in 100 ml flask containing 50 ml Y35N5D2iP liquid medium. The suspension cultures were incubated in the dark at 28ºC on a rotary shaker and agitated at 120 rpm. Half of the Y35N5D2iP liquid medium in the flask culture was discarded and replaced with fresh medium about every 14 days.

II.2.6.2 Protoplast isolation and purification

Protoplasts were isolated from embryogenic cells suspension up to about 14 days after fresh medium was added. The embryogenic cells suspension was collected by filtration with 300 µm nylon mesh and 0.5 g embryogenic cells was transferred into a 50 ml centrifuge tube containing 15 ml of filter-sterilized Ezm Sol VI (Table II.1). The embryogenic cells were resuspended in enzyme solution by inverting the centrifuge tube for 6-10 times. The centrifuge tube was placed in a horizontal condition and incubated in the dark without shaking at 26ºC for about 14 hours. After incubation, the mixture was diluted with 15 ml of filter-sterilized Wash Sol IV (Table II.2). The diluted mixture was resuspended by inverting the centrifuge tube 3-5 times and then was filtered through a sterilized double layer miracloth (22 µm) by collection in a 50 ml centrifuge tube. The filtration step was repeated 2-3 times until all the undigested tissues, cell clumps and cell wall debris were removed. The centrifuge tube was centrifuged at 60 ×g for 5 minutes at 22ºC and the supernatant was removed. The protoplasts pellet was resuspended by inverting the tube with addition of 10 ml Wash Sol IV, and then was centrifuged. After repeating 3 times with the washing step, the supernatant was removed completely, and the protoplasts pellet was resuspended with 5 ml filter-sterilized Rinse solution consisting of 3 % (w/v) KCl and 3.6 % (w/v) mannitol at pH 5.6. The yield and viability of the purified protoplasts were calculated as previous described (II.2.5.3). Cell wall formation was evaluated after stained by fluorescent brightener 28 (Sigma).

II.2.7 Oil palm protoplasts culture

II.2.7.1 Media optimization

The purified protoplasts were cultured using different media (Appendix I) either in liquid or embedded in agarose solidified media. Five ml rinse solution containing the purified protoplasts was allowed to settle for 20 minutes at room temperature. For liquid culture, the rinse solution was replaced with liquid media and 2 ml each dispensed into 24 wells culture plate. For agarose solidified cultures, the protoplasts pellet was resuspended with a double concentration of liquid media at the density 2 x 10^5 protoplasts/ml. Agarose sea plaque (Duchefa) was dissolved at the concentration of 1.2 % (w/v) by heating in distilled water containing of 0.1 % (w/v) 2-N-morpholino ethane sulfonic acid (MES), and then the pH was adjusted to 5.7. The agarose solution was filter-sterilized and kept at 37ºC. Equal volumes of protoplasts suspension and agarose were mixed by adjusting the final concentration to 0.6 % (w/v) of agarose, and then 2 ml each of the mixtures was dispensed into 24 wells culture

plate. The culture plate was placed at room temperature for an hour for agarose solidification. The protoplasts embedded in agarose solidified media in each well were covered with 500 µl of the same liquid media was used for preparation of agarose solidified cultures. The culture plates containing liquid or agarose solidified cultures were sealed and incubated at 28ºC in the dark. The culture was monitored microscopically everyday to observe the first and second cell division, and seven days intervals for microcolonies and microcalli formations.

II.2.7.2 Plant growth regulators (PGRs) optimization

The PGRs optimization was performed on agarose solidified cultures. All PGRs used were prepared at the concentration of 1 µM/µl and the pH was adjusted to 5.7. The filter-sterilized PGRs were added in 24 wells culture plate at different combinations and concentrations (Table II.3).

II.2.7.3 Agarose bead cultures

The mixture of protoplasts and agarose was prepared by using the same procedure for preparation of agarose solidified cultures with the exception that the protoplasts pellet was resuspended in Y3A liquid medium supplemented with the optimum PGRs and 0.6 % (w/v) agarose sea plaque. Agarose beads were prepared by dropping 200 µl of the mixture into a 35 x 10 mm petri dish. After agarose solidification, 10 ml of 21 % (w/v) osmoticum solution was added to petri dish and incubated at 28ºC in the dark for 3-5 days. Three types of osmoticum solutions were used: sucrose, glucose and mannitol. Each was dissolved in water, adjusting the pH to 5.7 and then filter-sterilized. The osmoticum solution was replaced with Y3A liquid medium in shaking condition at 50 rpm by refreshing the medium at 14-day intervals. When the formation of microcolonies was observed, Y3A liquid medium was changed to Y3 liquid medium and the agarose beads were cultured until the microcalli were detected. After the microcalli appeared visible by eye, the agarose beads was cultured in Y35N5D2iP liquid medium supplemented with different concentrations of ascorbic acid (AA: 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l and 400 mg/l), silver nitrate (AgNO3: 5 mg/l, 10 mg/l, 15 mg/l) and activated charcoal (AC: 0.1 g/l, 0.3 g/l, 0.5 g/l and 1.0 g/l). The cultures were continued until the microcalli growth to embryogenic calli.

II.2.7.3 Division, microcolonies and microcalli frequencies

Protoplast division frequency was calculated by counting the number of protoplasts divided by the total number of protoplasts in one representative microscope field. Three microscopic fields were averaged to represent one experiment in which the experiment was performed in 3 replicates to give an average range of protoplast division frequency. A similar calculation procedure was used for microcolonies and microcalli formation frequencies.

II.2.7.4 Plant regeneration of protoplasts-derived embryogenic calli

The agarose beads were transferred to a Y3 solid medium [0.6 % (w/v) plant agar] when microcalli developed to 5-10 mm in size of whitish and yellowish embryogenic calli. The agarose beads were maintained on Y3 solid medium supplemented with different concentrations of a combination of NAA (0.5-10 μM) and BA (0.1-5 μM) until the formation of embryos was observed. The agarose beads containing the embryogenic calli were incubated at 28°C in the dark and were subcultured every 30 days in fresh medium. The embryos were transferred onto ECI solid medium supplemented with the optimum PGRs of NAA and BA, and then were incubated at 28°C in the light until small plantlets were produced. Small plantlets were transferred onto EC1 solid medium supplemented with 0.1 μM NAA for root formation and plant regeneration.

II.2.8 Oil palm protoplast transformation

II.2.8.1 Polyethylene glycol (PEG) mediated transformation

Protoplasts were isolated from a 3 month old suspension cultures at 7 and 14 days after subculture following the protocol described (II.2.6.2). After twice washing with Wash Sol IV, the supernatant was mostly removed leaving about 1 ml washing solution and incubated at room temperature for 10 minutes. The protoplasts suspension was then incubated at 45ºC for 5 minutes and immediately placed on ice for 1 minute, then incubated at room temperature for 10 minutes. A 500 μl aliquot of the protoplasts suspension was then placed as a single droplet in the middle of a 60 mm x 15 mm petri dish (no. 628102, Greiner Bio-One, Germany). The protoplasts drop was surrounding by 5 drops of 100 μl PEG-MgCl solution containing 25-50 % (w/v) PEG 4000, 10-100 μM $MgCl_2.6H_20$ which were dissolved in Rinse solution adjusted to pH 6.0. Twenty five or 50 μg of plasmid DNA was added slowly to the protoplasts drop, mixed by stirring with 200 μl tip and incubated at room temperature

in the dark. After incubation for 10-30 minutes, the DNA-protoplasts drop was sequentially mixed with each of PEG-MgCl drop by stirring with 200 µl tip and incubated for another 30 minutes, then 4 ml Wash Sol IV was added drop by drop and incubated in the dark at 26ºC for 9 days.

II.2.8.2 Confocal laser scanning microscopy (CLSM)

Protoplasts were observed using a CLSM (Leica TCS 5 SP5 X) and visualized by Leica Microsystem LAS AF. GFP and autofluorescence of the chlorophyll were excited at 488 nm and 543 nm wavelengths, respectively. The emission filters were 500-600 nm and 675-741 nm for chlorophyll autofluorescence. PEG-mediated transfection efficiency was calculated as the percentage of the number of protoplast fluorescing green (GFP positive protoplasts) divided by the total number of protoplasts in one representative microscope field. The calculation was performed three times for a total of not less than 200 protoplasts.

II.2.9 DNA microinjection mediated transformation

II.2.9.1 Protoplast isolation

Protoplasts were isolated from a 7 day subculture of a 3 month old suspension culture as described (II.2.6.2). After twice repeating the washing step, the supernatant was mostly removed leaving 3 ml washing solution. The centrifuge tube containing the protoplasts suspension was incubated in a vertical position in the dark for 24 hours at 28ºC. After incubation, the protoplasts suspension was diluted by 10 ml of Rinse solution and resuspended by inverting the centrifuge tube for 3-5 times, and then centrifuged at 60 xg for 5 minutes at 22ºC. After repeating the rinsing step, the supernatant was removed completely and the protoplasts pellet was embedded with 3 ml filter-sterilized alginate solution consisted of 1 % (w/v) alginic acid sodium salt (A2158, Sigma) dissolved in Y3A liquid medium [5.5 % (w/v) sucrose and 11.9 % (w/v) glucose supplemented with 10 µM NAA, 2 µM 2.4-D, 2 µM IBA, 2 µM GA3, 2 µM 2iP and 200 mg/l ascorbic acid] adjusted to pH 5.6, which the Y3 macroelements was prepared without calcium chloride ($CaCl_2.2H_2O$).

II.2.9.2 Alginate thin layer preparation

Alginate-embedded protoplasts were distributed as a thin layer onto supporting media comprising 1.5 ml filter-sterilized Y3A [5.5 % (w/v) sucrose and 11.9 % (w/v) glucose

supplemented with 0.1 % (w/v) $CaCl_2.2H_20$] solidified with 1 % (w/v) agarose sea plaque, in 35 mm x 10 mm petri dish (no. 627161, Greiner Bio-One, Germany). The distribution of alginate-embedded protoplasts was performed by dropping 100 µl alginate-embedded protoplasts at the edge of petri dish and immediately held the petri dish at an angel of 35º to allow the drop distributed as a thin layer. The dishes were placed horizontally into 94 mm x 15 mm two compartment dishes (no. 635102, Greiner Bio-One, Germany) where the alginate solidified within 1-2 minutes. Three ml sterile water was added into the outer compartment in order to prevent the alginate layer from drying out. The plates were sealed and incubated at 28ºC in the dark for 3 days.

II.2.9.3 Microinjection workstation

The microinjection workstation consisted of a Leica DM LFS upright microscope (Leica Microsystems Wetzlar GmbH, Germany) with a joystick controlled motorized objective revolver for HCX APOL U-V-I water immersion objectives (10x, 20x, 40x and 63x), mounted on a fixed table and placed in a laminar. The microscope was equipped with a Luigs and Neumann Manipulator set with a control system SM-5 and SM-6 (Luigs and Neumann, Germany).

II.2.9.4 Preparation of DNA injection solution

Plasmid DNA was prepared by midi scale Plasmid DNA Purification Kit (NucleoBond PC100; MACHEREY-NAGEL, Germany) and was dissolved at concentration of 1 µg/µl in sterile water. The plasmid was restricted with *Hind*III and *Eco*RI to yield the CFDV-hrGFP-nos cassette as a 1.5 kb fragment. The fragment was separated from the vector sequence (pUC19) by electrophoresis on a 1 % (w/v) agarose gel. The DNA fragment containing the cassette was excised using a clean blade and isolated using the PCR clean-up Gel extraction Kit (NucleoSpin Gel and PCR Clean-up) according to the manufacturer's description (MACHEREY-NAGEL, Germany). The DNA cassette was then diluted with sterile water to concentrations of 100 ng/µl. The DNA solution was mixed with Lucifer Yellow CH dilithium salt (L0259, Invitrogen) in a proportion of 10:0.1 and filter-sterilized using the Ultafree-MC filter (Durapore 0.22 um, type: GV; No. SK-1M-524-J8; Millipore) by spinning at 10,000 rpm, 15 minutes at 22°C. The eluted DNA were partitioned into 10 µl aliquots as DNA injection solution and stored at -20°C until required.

II.2.9.5 Loading the DNA injection solution into microinjection needle

The DNA injection solution was centrifuged at 14,000 rpm for 30 minutes at 4°C before loading into Femtotip II microinjection needle (no. 5242 957.000, Eppendorf). A 5 µl aliquot of DNA injection solution was loaded as close as possible to the tip of Femtotip II microinjection needle through back opening of the needle using microloader (no. 5242 956003, Eppendorf). After 30 minutes standing at room temperature, the needle was filled with sterile mineral oil (M8410, Sigma) using the microloader and tightly mounted in the capillary holder of microinjector CellTram vario (no. 5176 000.033, Eppendorf), and then fixed onto micromanipulator.

II.2.9.6 Microinjection of oil palm protoplasts

A plate containing alginate layer was placed on the microscope stage, and the vitality of embedded protoplasts was confirmed by using the 10x objective. The objective was raised to maximum position to freely allow the needle tip to reach the center of the field view with the X- and Y-axis controller (Control system SM-5) of the manipulator. The needle was lowered as close as possible to the alginate layer with the Z-axis controller and the cytoplasm or nucleus of target protoplast was identified by adjusting the 20x objective to optimal resolution and contrast, after which the needle tip was moved to right above the protoplast with the X- and Y-axis hand wheel controller. The needle tip was then inserted into the alginate layer just next to the protoplast by using Z-axis hand wheel controller and penetrated into the protoplast by using the X-axis hand wheel controller. The DNA injection solution was slowly injected into the protoplast by using a microinjector CellTram vario, which was confirmed by the fluorescence illumination. The needle tip was carefully withdrawn from the protoplast and moved to the next target protoplast. The injected protoplasts were monitor periodically by using Leica MZ16F fluorescent stereomicroscope with GFP3 filter (Leica Microsystems Wetzlar GmbH, Germany).

II.2.9.7 Alginate layer culture

Following microinjection, the plates containing the alginate layer were incubated in the dark at 28°C for 5 days. The alginate layers were then separated from supporting media and transferred into 60 mm x 15 mm petri dishes containing 3 ml Y3A liquid medium consisted of 5.5 % (w/v) sucrose and 8.2 % (w/v) glucose supplemented with 10 µM NAA, 2 µM 2,4-D, 2 µM IBA, 2 µM GA3, 2 µM 2iP and 200 mg/l ascorbic acid. The dishes were incubated

in the dark by shaking at 50 rpm at 28°C. After 2 weeks, the medium was replaced with similar Y3A liquid medium but the concentrations of sucrose and glucose were decreased to 4 % (w/v) and 7.2 % (w/v), respectively. The alginate layers were cultured in this medium for a month by refreshing the medium at 14-days intervals, then replaced with Y3A liquid medium comprising of 4 % (w/v) sucrose until the microcalli were observed.

III RESULTS

III.3.1 Transient expression PIPP, D12 and HSA in tobacco protoplasts

III.3.1.1 Construction of PIPP genes driven by LSP, MSP and KSP

The PCR products of 0.8 kb PIPP light chain gene (PIPPLc) and 1.5 kb PIPP heavy chain gene (PIPPHc), were amplified from the plasmid cPIPP with primers PIPPlight fw EcoRI and PIPPlight rev XhoI, PIPPheavy fw EcoRI and PIPPheavy rev XhoI, respectively. Then, the PCR products were cloned into pCRII-TOPO and the sequences integrity was confirmed by DNA sequencing. The *Eco*RI and *Xho*I restricted PIPPHc and PIPPLc fragments were used to produce LSP-PIPPHL, MSP-PIPPHL and KSP-PIPPHL vectors by following the strategy as illustrated in Figure III.1.

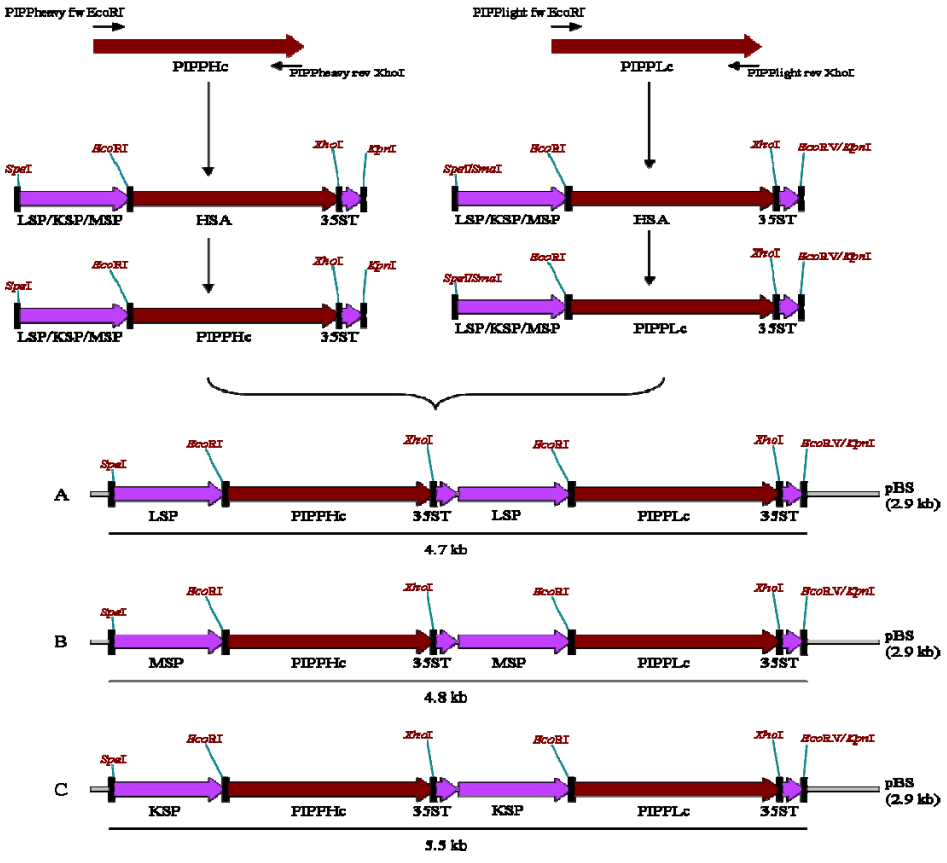

Figure III.1: Strategy for construction of PIPP genes vectors.

The PIPPHc fragment was inserted into LSP/KSP/MSP-HSA by replacing the HSA fragment to generate LSP/KSP/MSP-PIPPHc. LSP/KSP/MSP-PIPPLc were generated using similar strategy but the 35ST fragment contained *Eco*RV site at 3'end. Then *Spe*I and *Kpn*I-blunted restricted LSP/KSP/MSP-PIPPHc fragments were inserted into *Spe*I and *Sma*I site of LSP/KSP/MSP-PIPPLc to create LSP-PIPPHL (A), MSP-PIPPHL (B) and KSP-PIPPHL (C).

III.3.1.2 The functionality of PIPP, D12 and HSA gene constructs

The functionality of the PIPP, D12 (Appendix 2) and HSA (Appendix 3) constructs was verified by transient expression in tobacco protoplasts. Freshly isolated protoplasts were transfected with the constructs DNA using PEG-mediated transformation and the transfected protoplasts were subjected to total protein isolation. The extracted proteins were separated using SDS-PAGE and blotted onto a nylon membrane for immunodetection with appropriate antibodies. The results showed the protein bands were detected for all samples which were identical to positive control (lane P) but not for protein sample from untransfected protoplasts (lane wt) (Figure III.2).

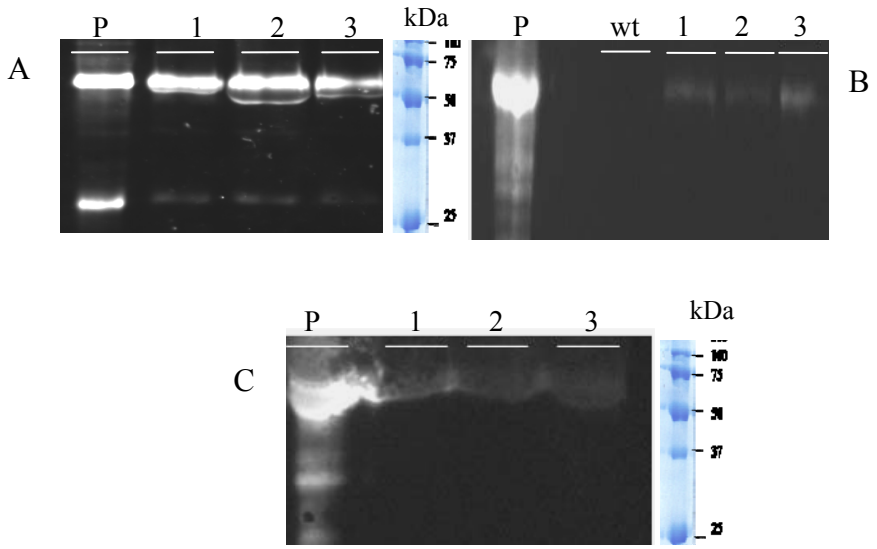

Figure III.2: Detection of the recombinant proteins of PIPP, D12 and HSA in transfected tobacco protoplast using specific antibodies.

(A) Detection of the PIPP heavy chain at ~55 kDa and the PIPP light chain at ~30 kDa. Lane 1: LSP-PIPPHL; Lane 2: MSP-PIPPHL; Lane 3: KSP-PIPPHL. (B) Detection of the D12 heavy chain at ~55 kDa and the D12 light chain at ~30 kDa. Lane 1: KSP-D12HL; Lane 2: MSP-D12HL, Lane 3: LSP-D12HL. (C) Detection of HSA at ~70 kDa. Lane 1: KSP-HSA, Lane 2: MSP-HSA, Lane 3: LSP-HSA. Lane P and Lane wt represented human serum as positive control and non-transfected protoplasts as negative control, respectively.

The transient expressions of PIPP and D12 genes in protoplasts were demonstrated by the detection of two protein band at 55 kDa and 30 kDa corresponding to the heavy and light chain. However, faint bands of light chain were observed for all samples suggesting the light chain has a different stability compared to the heavy chain. Meanwhile, a single band at 70 kDa was detected for samples protein from protoplasts transfected with HSA gene.

Subsequently, the results demonstrated that the PIPP, D12 and HSA genes were successful constructed and transfected into tobacco protoplasts, and then transiently expressed which showing the functionality of all constructs even when gene expression was driven by three different oil palm promoters, LSP, MSP and KSP.

III.3.2 Characterization of oil palm tissue-specific promoter in tobacco plants

III.3.2.1 GFP expression vectors

The construction of LSP-GFP$_{ER}$ (4.9 kb), KSP-GFP$_{ER}$ (5.3 kb), and MSP-GFP$_{ER}$ (5.0 kb) vectors was performed as shown in Figure III.3. First, the vectors were digested with *Spe*I and *Eco*RV to release DNA fragments of 2.0 kb LSP-GFP$_{ER}$, 2.4 kb KSP-GFP$_{ER}$ and 2.1 kb MSP-GFP$_{ER}$, which then were inserted into *Sma*I site of pBin19 (11.8 kb; Appendix 4) by blunt end ligation to generate GFP expression vectors, LSP-GFP$_{ER}$-pBin19, KSP-GFP$_{ER}$-pBin19 and LSP-GFP$_{ER}$-pBin19.

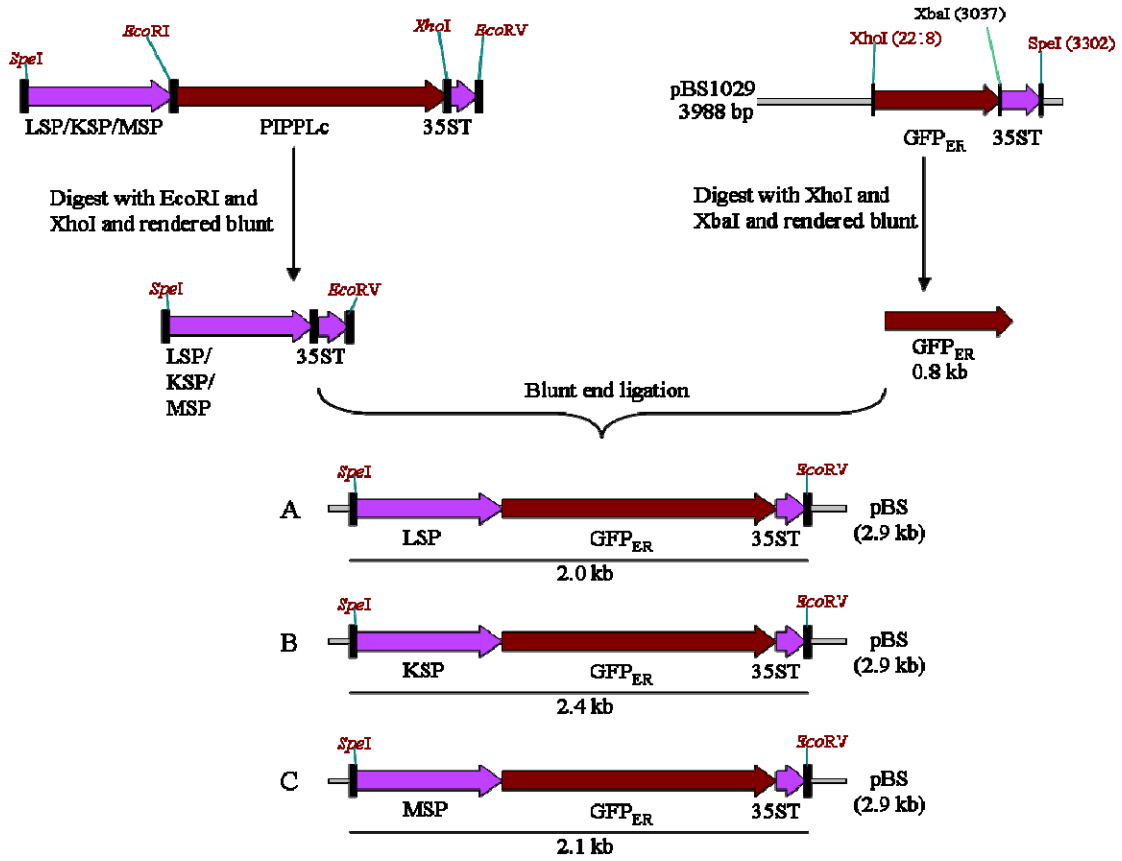

Figure III.3: Strategy for construction of GFP expression vectors.

Arrows in DNA fragments indicate the orientation of each gene assembled in LSP-GFP$_{ER}$ (A), KSP-GFP$_{ER}$ (B) and MSP-GFP$_{ER}$ (C) vectors.

III.3.2.2 GFP expression pattern in transgenic tobacco plants

The tissue specificity of oil palm tissue-specific promoters, LSP, MSP and KSP promoters was verified in a heterologous plant system by expressing an ER targeted GFP gene in tobacco plants. The constructs LSP-GFP_{ER}-pBin19, MSP-GFP_{ER}-pBin19 and KSP-GFP_{ER}-pBin19 were transferred into the *A. tumefaciens* LBA4404 strain and then transformed into tobacco plants using leaf-disc transformation. After selection on kanamycin media, 5 independent transgenic lines for the constructs, LSP-GFP_{ER}-pBin19 and KSP-GFP_{ER}-pBin19, and 4 independent transgenic lines for the construct MSP-GFP_{ER}-pBin19 were identified by PCR (Figure III.4).

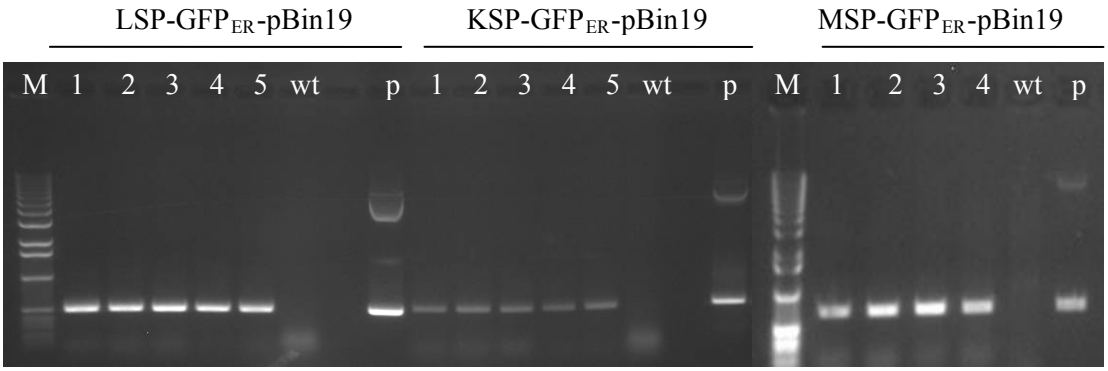

Figure III.4: Analysis of GFP_{ER} transgenic tobacco plants by PCR.

The genomic DNA of LSP-GFP_{ER}-pBin19, MSP-GFP_{ER}-pBin19 and KSP-GFP_{ER}-pBin19 transgenic tobacco plants was used as template for PCR amplification of 0.5 kb part of LSP-GFP genes using primers LSP550fw and GFPbw, 0.4 kb KSP gene using primers KPESmaIfw and KPErevraus, and 0.7 kb part of MSP-GFP genes using primers MSPfw410 and GFPbw, respectively. The numbers indicates the genomic DNA from independent transgenic lines. M: 1 kb DNA ladder, p: plasmid DNA.

Different parts of T0 transgenic tobacco plants (five months old) were cut with a razor blade and analyzed by CLSM. The GFP fluorescence was observed in all green tissue of LSP-GFP_{ER} transgenic tobacco plants, such in the epidermal and parenchyma cells of leaves and also stem (Figure III.5: A-C). Furthermore, no GFP fluorescence was found in non-green tissue such as root and flower. For KSP-GFP_{ER} transgenic tobacco plants, no GFP fluorescence was observed in all tissue of the plant. Meanwhile, for MSP-GFP_{ER} transgenic tobacco plants, GFP fluorescence with low intensity was detected in epidermal cells of leave tissues (Figure III.5: D-E) indicating the possibility of MSP promoter regulation in tobacco leaves.

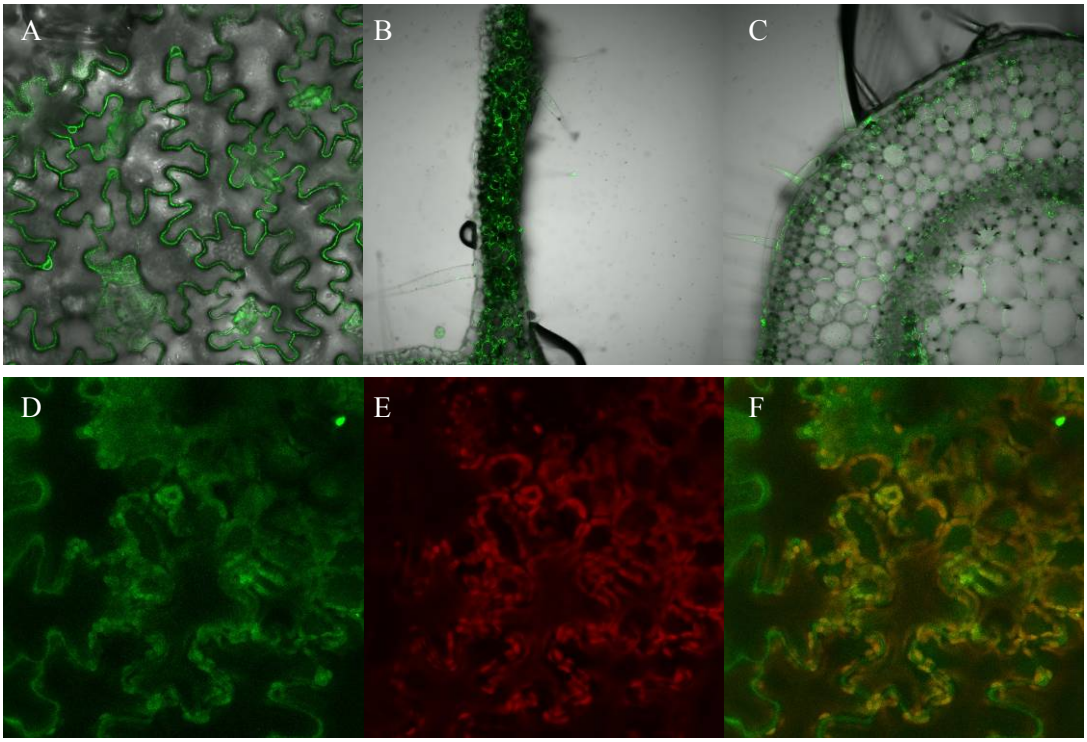

Figure III.5: GFP fluorescence in transgenic tobacco plants.

GFP and bright merged images of GFP fluorescence in the epidermal cells (A), parenchyma cells (B) and stem (C) of LSP-GFP_{ER} transgenic tobacco leaves. GFP fluorescence in epidermal cells (D: GFP image; E: autofluorescene image; F: merged image) of MSP-GFP_{ER} transgenic tobacco leaves.

Further CLSM analysis of T1 LSP-GFP_{ER} transgenic seedlings (two weeks after germination) revealed a similar expression pattern as in T0 LSP-GFP_{ER} transgenic tobacco plants, which GFP fluorescence was observed in all green tissues (Figure III.6) but not in seeds and roots. Meanwhile, since KSP and MSP promoters are regulated in kernel (endosperm) and mesocarp tissue of oil palm, it is expected that the GFP gene will be expressed in KSP-GFP_{ER} and MSP-GFP_{ER} transgenic seeds. Unfortunately, GFP fluorescence was not observed in T1 seed or all tissues of seedling from KSP-GFP_{ER} transgenic tobacco plants. Similarly, observation on the T1 seeds or seedlings from MSP-GFP_{ER} transgenic plants did not show any GFP fluorescence.

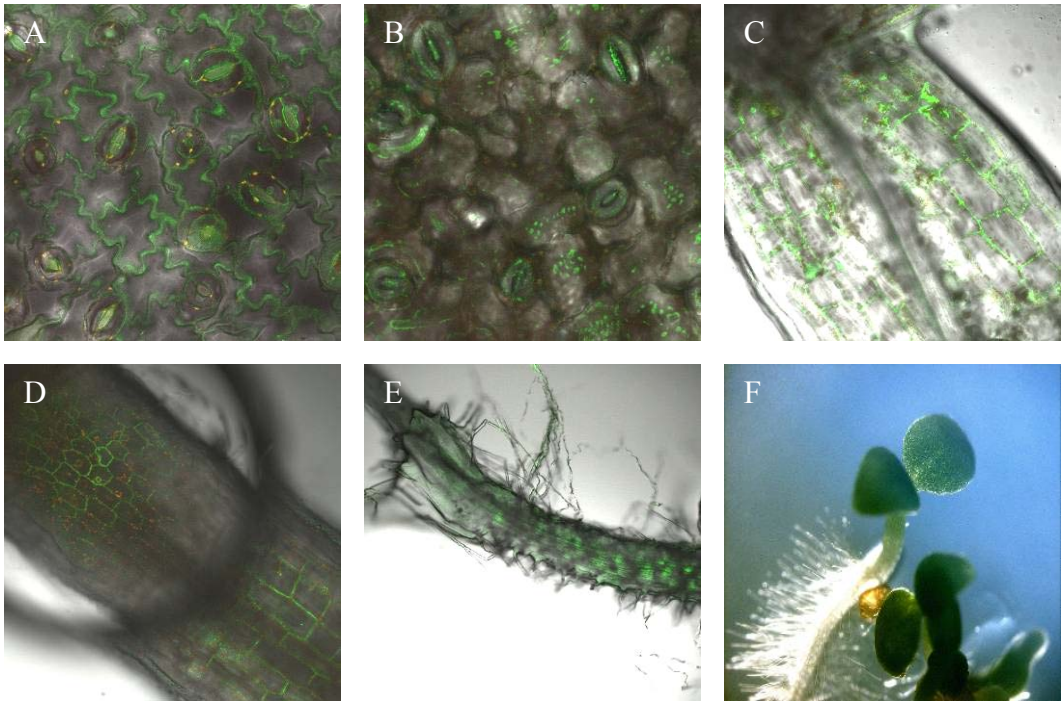

Figure III.6: GFP fluorescence in different tissues of LSP-GFP$_{ER}$ transgenic seedlings.

The merged images of GFP fluorescence in the lower epidermal cells (A), upper epidermal cells (B), epicotyl cells (C), petiole cells (D) and radicle cells (E). Seedling observed under binocular microscopy is shown in F.

III.3.3 Stable expression of PIPP, D12 and HSA in tobacco plants

III.3.3.1 Plant expression vectors

The LSP-hyg-LSP-PIPPHL vector was constructed as shown in Figure III.7. Therefore, the *Eco*RI and *Xho*I restricted PIPPHc fragment was inserted and replaced the *Eco*RI and *Xho*I restricted HSA fragment of LSP-hyg-LSP-HSA to generate LSP-hyg-LSP-PIPPH vector. Then LSP-hyg-LSP-PIPPH vector was digested with *Spe*I and *Sma*I as a DNA vector for blunt end ligation with LSP-PIPPL fragment which was digested with *Spe*I and *Kpn*I, to produce LSP-hyg-LSP-PIPPHL vector (9.9 kb). For the construction of LSP-hyg-LSP-PIPPHL-0380 vector, the 7.0 kb LSP-hyg-LSP-PIPPHL fragment was cleaved from LSP-hyg-LSP-PIPPHL vector by *Spe*I and *Eco*RV digestion, and then cloned into the *Sma*I site of pCambia0380 (6.8 kb) by blunt end ligation. On the other hand, the *Sac*II and *Kpn*I restricted 7.2 kb LSP-hyg-LSP-D12HL fragment (Appendix 5) and 5.8 kb LSP-hyg-LSP-HSA fragment (Appendix 5), were inserted into *Sma*I site of pCambia0380 to produce LSP-hyg-LSP-D12HL-0380 and LSP-hyg-LSP-HSA-0380 vectors, respectively.

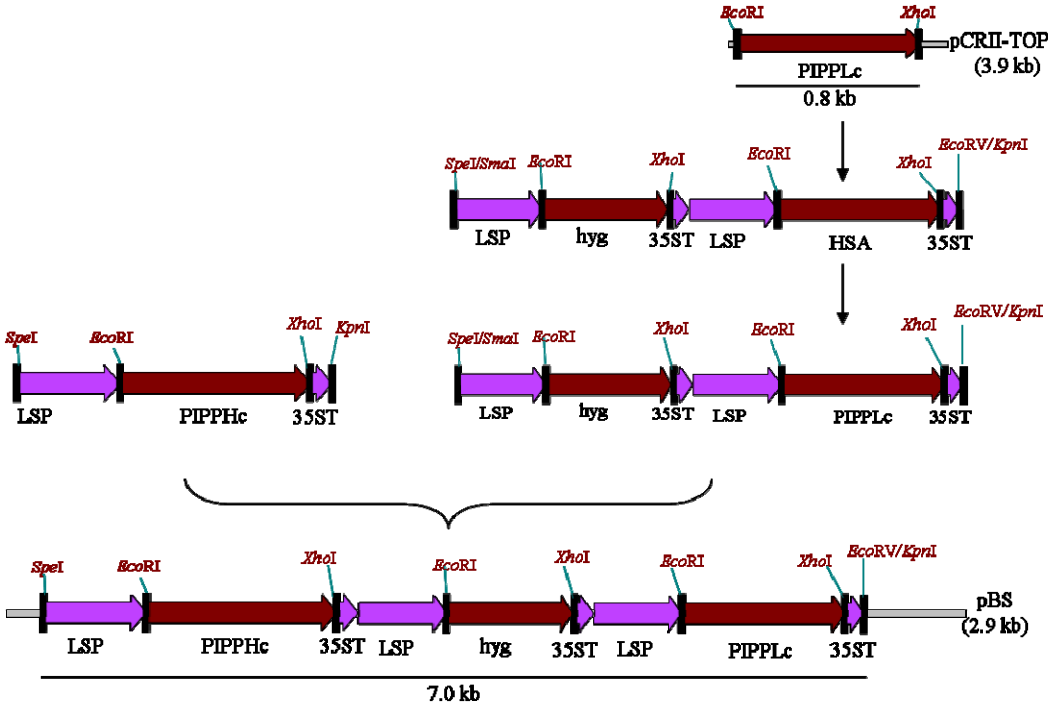

Figure III.7: Strategy for the construction of LSP-hyg-LSP-PIPPHL vector.

Arrows indicate DNA fragments assembled in the construct.

III.3.3.2 Transformation and regeneration transgenic tobacco plants

The LSP-hyg-LSP-PIPPHL-0380, LSP-hyg-LSP-D12HL-0380 and LSP-hyg-LSP-HSA-0380 vectors were transferred into the *A. tumefaciens* LBA4404 strain by electroporation. Five putative colonies from each transformation were directly used for PCR amplification to select for positive clones. All clones were confirmed to carry the hyg gene indicating the constructs were successfully transferred into Agrobacteria. The positive clones were transformed into tobacco plants using leaf-disc transformation.

The first attempt to regenerate transgenic tobacco plants using 50 ug/l concentration of hygromycin selection media failed, which was indicated by the lack in callus formation after two months. Therefore, the concentration of hygromycin was reduced to 10 ug/l, which resulted in the formation of hygromycin resistance calli after a month of cultivation. The hygromycin resistance calli developed at the cuts of leaf pieces from the transformation for all constructs (Figure III.8: A-C). Shoots were obtained from transgenic calli within two months after transformation (Figure III.8: D-F) and individual shoots from each callus line were transferred to the glass cultivation flask containing hygromycin selection media for

further growth of plantlets (Figure III.8: G) and roots (Figure III.8: H). However, the roots hardly developed indicating the hyg gene driven by LSP promoter was not active in the root tissue. Thus, the plantlets were transferred onto media without selection. After a month, the plants with roots (Figure III.8: I) were transferred to soil for development of true plants. The plants obtained from transformation with the constructs LSP-hyg-LSP-PIPPHL-0380, LSP-hyg-LSP-D12HL-0380 and LSP-hyg-LSP-HSA-0380 were designated as T0PIPP, T0D12 and T0HSA plants, respectively. In total, nearly seven months were required for the transformation and regeneration of transgenic tobacco plants using the hyg gene under the control of LSP promoter.

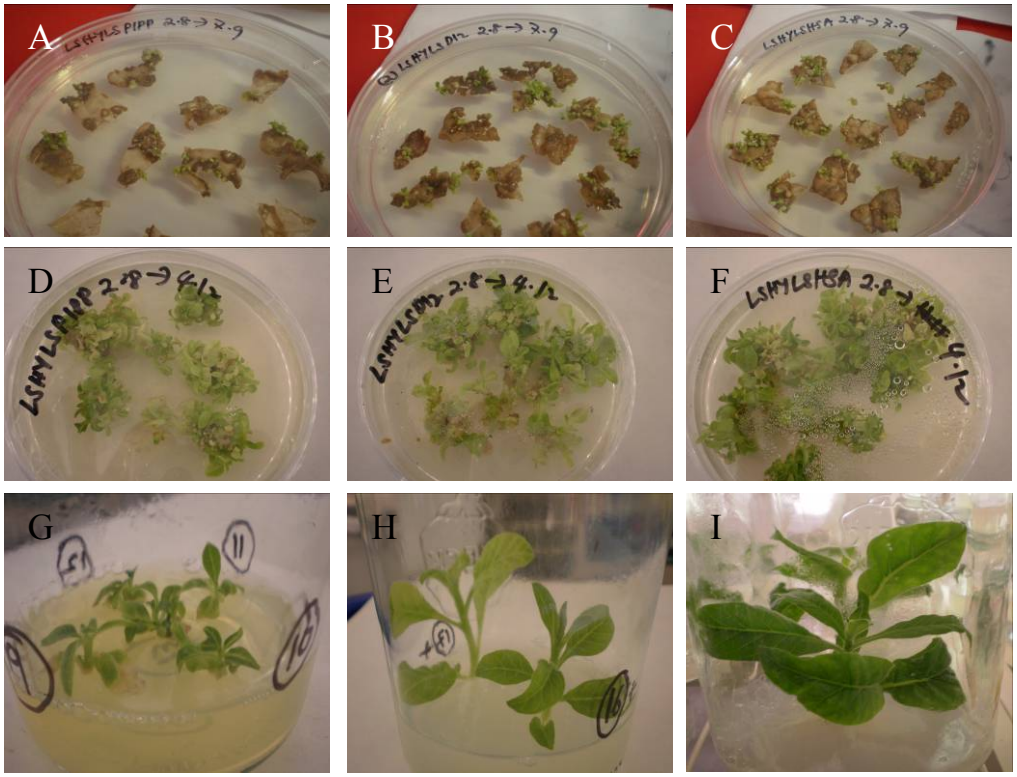

Figure III.8: Transformation and development of transgenic tobacco plants using hygromycin selection.

The development of hygromycin resistance calli (A-C), shoots (D-F), small plantlets (G), plantlets (H) and young plants with roots cultured on media without selection (I). Calli and shoots derived from transformation with plant expression vectors of LSP-hyg-LSP-PIPPHL-0380, LSP-hyg-LSP-D12HL-0380 and LSP-hyg-LSP-HSA-0380 are shown in A and D, B and E, C and F, respectively.

III.3.3.3 Identification of transgenic tobacco plants by PCR analysis

The T0 transgenic tobacco plants with hygromycin resistance were screened for positive clones by PCR. Since all the transformed plants were derived from plants transformed with T-DNA region carrying hyg gene driven by LSP promoter, the amplification of LSP and hyg gene using primers, LSP550fw and Hyrevvornraus, was first used for further verification of transgenic status. The genomic DNA was extracted from 7, 8 and 14 of independent putative transgenic T0PIPP, T0D12 and T0HSA plantlets (four months old) respectively, and used as a template for PCR analysis (Figures III.9).

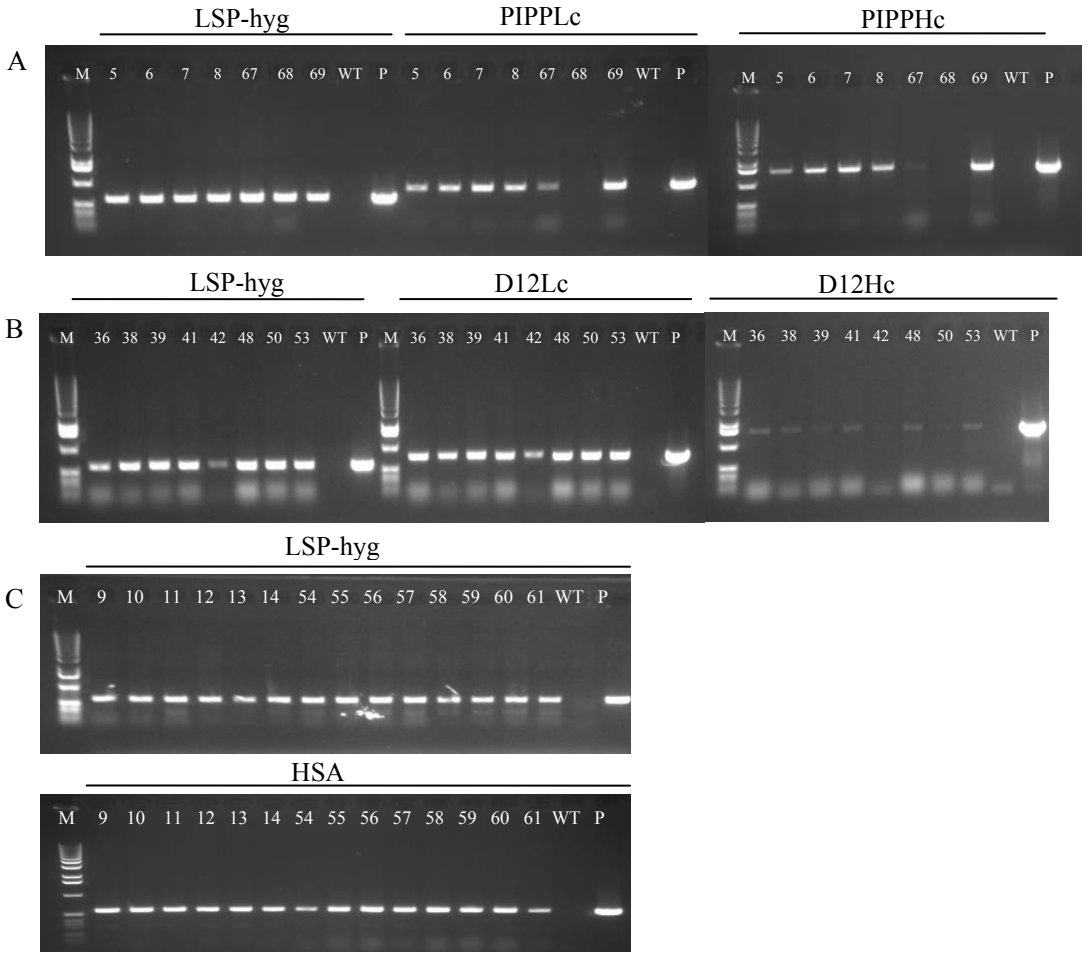

Figure III.9: Molecular characterization of transgenic tobacco plants.

PCR on genomic DNA of T0PIPP (A), T0D12 (B) and T0HSA (C) transgenic plants using corresponding primers for amplification of a part of LSP-hyg (0.6 kb), PIPPLc (0.8 kb), PIPPHc (1.4 kb), D12Lc (0.7 kb), D12Hc (1.5 kb) and a part of HSA (0.5 kb) genes. The numbers indicates the genomic DNA from independent transgenic lines. Lane P: plasmid DNA as positive control. Lane WT: genomic DNA of wild type plant. Lane M: 1 kb DNA ladder.

Results showed that all samples were positive for the presence of a part of LSP and hyg gene (~0.6 kb; Figure III.9: A-C). The PCR product was not observed for the untransformed plant (lane wt), while the positive control (lane P) produced a PCR product of the same size (~0.6 kb). The presence of PIPP, D12 and HSA genes in all samples were further confirmed by using primers PIPPlightfwEcoRI and PIPPlightrevXhoI for 0.8 kb PIPP-light chain gene, PIPPheavyfwEcoRI and PIPPheavyrevXhoI for 1.4 kb PIPP-heavy chain gene, D12lightfwEcoRI and D12lightrevXhoI for 0.7 kb D12-light chain gene, D12heavyfwBspHI and D12heavyrevXbaI for 1.5 kb D12-heavy chain gene, HSAfw and HSArevraus for 0.5 kb HSA gene (Figure III.9: A-C). The results of the PCR analysis demonstrated that all genes in T-DNA regions were successful integrated into the tobacco plant genome.

III.3.3.4 Characterization of transgenic tobacco plants

In order to confirm the expression of PIPP, D12 and HSA genes in transgenic tobacco plants on transcript level, RT-PCR experiments were carried out with total RNA. The total RNA was isolated from the young leaves of T0 transgenic plants (five months old) which showed positive results in the PCR amplification for PIPP, D12 and HSA genes. The integrity of RNA was verified by gel electrophoresis. Two ugs of total RNA from each sample were reverse transcribed to cDNA which then was used as a template (2 ul) for PCR amplification. The GAPDH endogenous gene using primers GAPDH fw and GAPDH rev was used as a positive control to amplify a 1.1 kb from genomic DNA and 0.3 kb from cDNA (Figures III.10 and III.11). Results showed that a 0.3 kb PCR product was amplified for all samples indicated that the cDNA was successful synthesized without genomic DNA contamination, which should amplified a 1.1 kb PCR product (lane g). The expression level was evaluated by comparison of the intensity of PCR band of GAPDH gene. The expression level of PIPP and D12 genes was verified using primers, PHC01-F and PHC02-R for PIPP-heavy chain gene, PLC01-F and PLC02-R for PIPP-light chain gene, D12Lc-Fw and D12Lc-Bw for D12-heavy chain gene, D12Hc-Fw and D12Hc-Bw for D12-light chain gene, which resulting in an amplification of 0.5 kb PCR products (Figure III.10). In general, the expression of heavy chain genes were higher than the light chain genes in all samples with the exceptions of transgenic lines 48, 50 and 53 from T0 D12 tobacco plants.

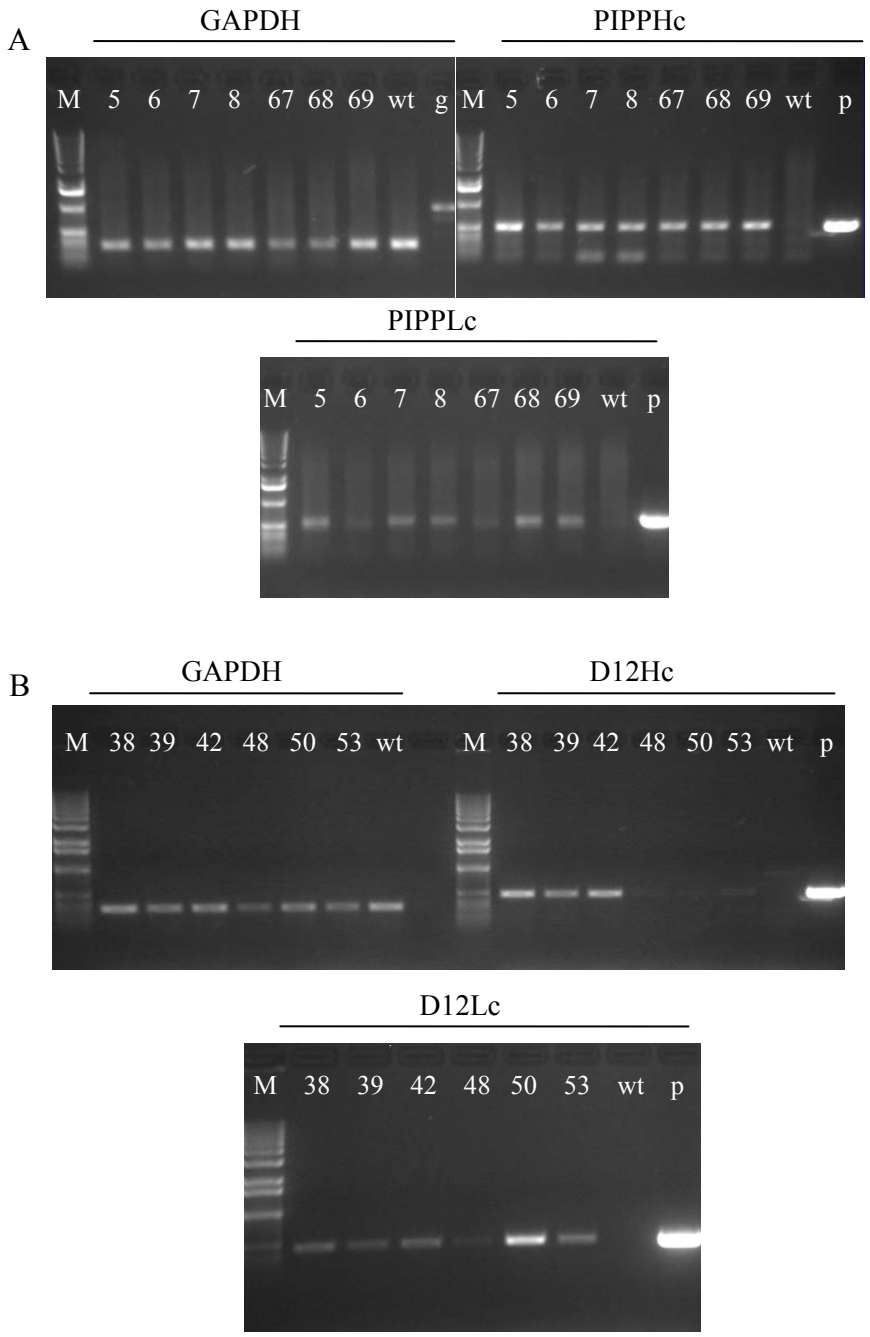

Figure III.10: Transgene expressions of T0PIPP and T0D12 transgenic tobacco plants.

The expression levels of the housekeeping GAPDH gene (0.3 kb), a gene fragment encoding the PIPPHc (0.5 kb) and a gene fragment encoding the PIPPLc (0.5 kb) from independent T0PIPP transgenic tobacco plants (A), and the housekeeping GAPDH gene (0.3 kb), a gene fragment encoding the D12Hc (0.5 kb) and a gene fragment encoding the D12Lc (0.5 kb) from independent T0D12 transgenic tobacco plants (B). Numbers indicates cDNA of independent transgenic lines. M: 1 kb DNA ladder; g: genomic DNA of wild type plant; wt: cDNA of wild type plant; p: plasmid DNA as positive control.

The expression level of HSA gene were low in all samples, especially for transgenic line 9, 11 and 12, for which the 0.5 kb PCR products were amplified using primers HSA-Fw and HSA-Bw (Figure III.11).

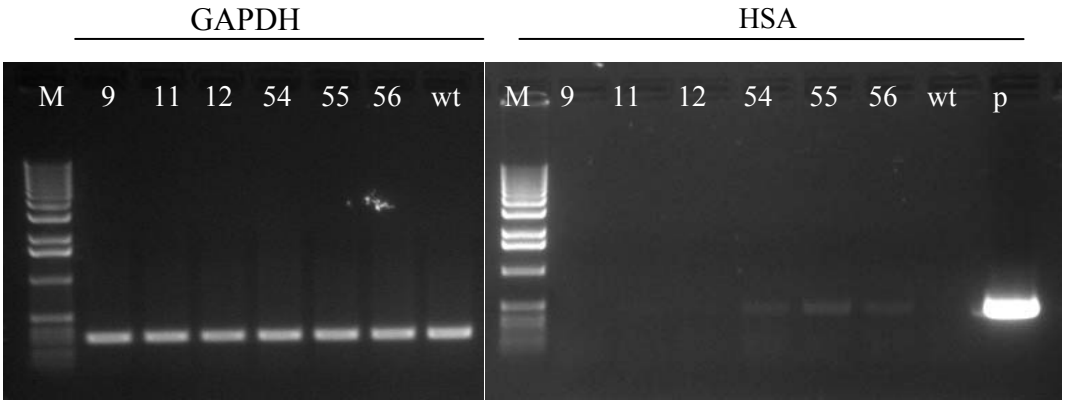

Figure III.11: Transgene expression of T0HSA transgenic tobacco plants.

The expression levels of the housekeeping GAPDH gene (0.3 kb), a gene fragment encoding the HSA (0.5 kb) from independent transgenic tobacco plants. Numbers indicates cDNA of independent transgenic lines. M: 1kb DNA ladder; wt: cDNA of wild type plant; p: plasmid DNA as positive control.

Since various expression levels were observed on transcript level by RT-PCR, the expression of PIPP, D12 and HSA genes in transgenic tobacco plants were further characterized on the protein level by western blot analysis. The total proteins were extracted from mature leaves of the same independent transgenic tobacco lines (seven months olds). Fifty mgs of the extracted proteins were separated using SDS-PAGE and blotted onto a nylon membrane for immunodetection with appropriate antibodies. In contrast to the results of RT-PCR, almost equal amounts of recombinant proteins were detected although the total protein samples were extracted from different transgenic lines (Figure III.12). Two clearly distincs bands identical to the commercial normal human serum (lane P) at 55 kDa and 30 kDa. The bands at 55 kDa and 30 kDa corresponding heavy and light chain of PIPP or D12 protein were detected for all transgenic lines (Figure III.12: A and B). For total protein samples from transgenic lines expressing HSA gene, only a single band at 70 kDa was detected identical to the commercial normal human serum as shown in Figure III.12: C. Additional faint bands were also detected for all samples of T0PIPP and T0HSA. This is not due to cross reaction of antibody since no band was detected for the protein sample from untransformed plant (lane wt). Therefore, the faint bands could be due to the degradation of protein samples during the extraction process.

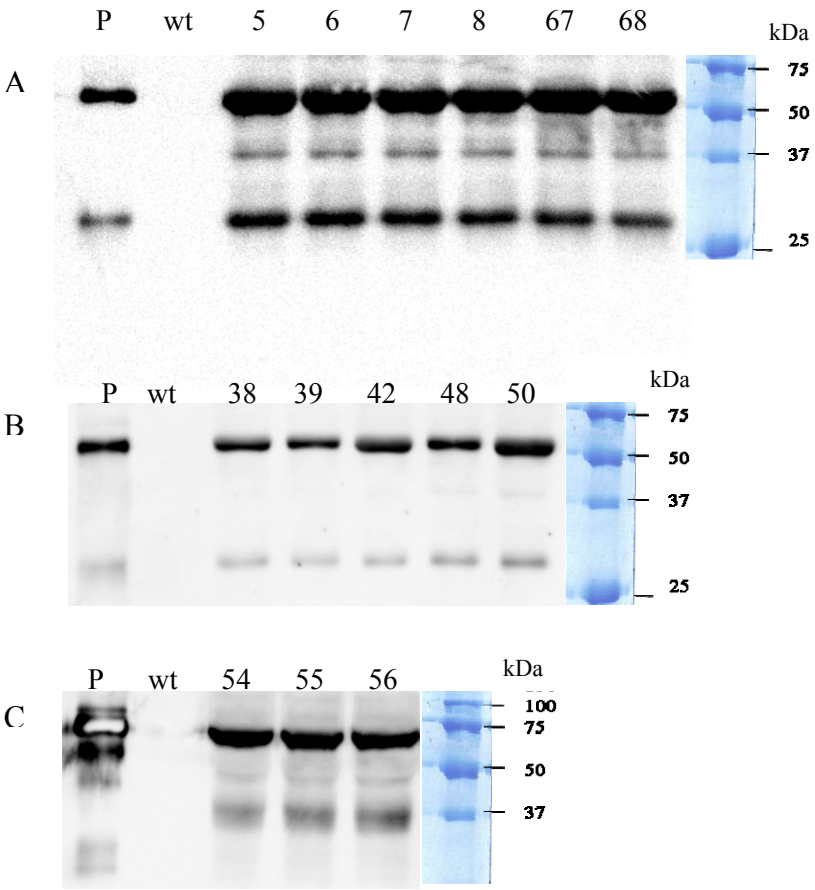

Figure III.12: Western blots of transgenic tobacco plants producing recombinant protein.

Blots were hybridised with immuno pure antibody-antigen human IgG (H+L) for PIPP of total protein of T0PIPP transgenic plants (A) and D12 of total protein of T0D12 transgenic plants (B), and monoclonal HSA antibody (Sigma) for HSA of total protein T0HSA transgenic plants (C). Numbers indicates the total protein from independent transgenic plants. Lanes are P: Human serum as a positive control, wt: protein extract from untransformed plant as negative control.

III.3.4 GFP expression in oil palm embryogenic calli

The activity of LSP promoter in oil palm embryogenic calli was verified by transient expression of the hrGFP gene. The LSP-GFP construct was bombarded into embryogenic calli using a biolistic approach. In parallel, the constructs CFDV-GFP and Ubi-GFP were also bombarded, which served as control. After three days of bombardment, the GFP fluorescence was observed in the samples bombarded with the constructs CFDV-GFP (Figure III.13: A) and Ubi-GFP (Figure III.13: B), but not for samples bombarded with LSP-GFP. This result demonstrated the LSP promoter was not active in oil palm embryogenic calli.

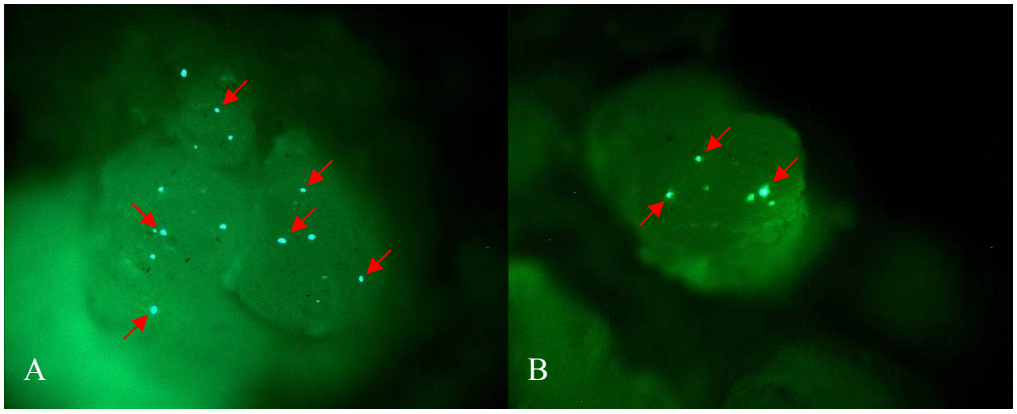

Figure III.13: GFP fluorescence in cells of the oil palm embryogenic calli.

GFP fluorescence in the embrogenic calli bombarded with CFDV-GFP (A) and Ubi-GFP (B) which was observed under fluorescence microscopy. GFP fluorescent cells are indicated by red arrows.

In order to determine the levels of expression of light-harvesting chlorophyll a/b binding protein (LHCB) gene for LSP promoter, fruit-specific type 3 metallothionein-like gene (MT3-A) gene for MSP promoter and glutelin (KT21) gene for KSP promoter, RT-PCR was performed for various tissues of oil palm. Total RNA was isolated from mature leaf, mesocarp, kernel, young leaf, spear, root and embryogenic calli and converted to cDNA. The endogenous actin gene was used as a control to amplify 0.6 kb from genomic DNA and 0.4 kb from cDNA (Figures III. 14: ACT). The expression level of LHCB gene was high in the mature leaf and young leaf and low in the mesocarp and spear (Figure III.14: LHCB). Meanwhile, high expression level was detected in mesocarp for MT3-A gene and surprisingly, the gene was also expressed at low level in mature leaf and high level in young leaf (Figure III.14: C). For KT21 gene, high level expression was detected in kernel and embryogenic calli (Figure III.14: KT21). The results indicates the expressions of LHCB, MT3-A and KT21 genes were not restricted in the corresponding tissue which shows the promoters of those genes will be regulated accordingly.

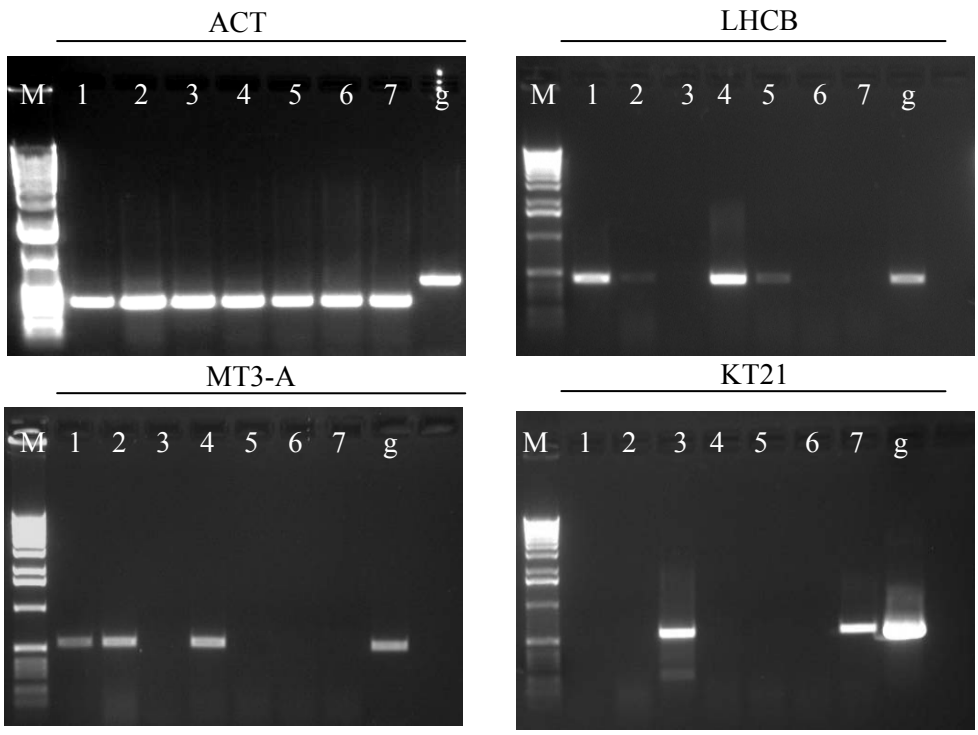

Figure III.14: Expression levels of the genes for LSP, MSP and KSP.

The expression levels of the housekeeping ACT gene (0.4 kb), a gene fragment encoding the LHCB (0.4 kb), a gene fragment encoding the MT3-A (0.5 kb), a gene fragment encoding KT21 (0.6 kb) in various tissues of oil palm (Lane 1: mature leaf, Lane 2: mesocarp, Lane 3: kernel, Lane 4: young leaf, Lane 5: spear, Lane 6: root, Lane 7: embryogenic calli). Genomic DNA of oil palm mature leaf as positive control (Lane g).

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III.3.5 Optimization of protoplast isolation from oil palm suspension culture

The effect of differently composed enzyme solutions on the efficiency of oil palm protoplast isolation was evaluated by incubating cells suspension in 5 different enzyme solutions (Table II.1). The components of enzyme solution were based on the Enz Sol 1, which has been reported by Sambanthamurthi *et al.* (1996). The highest yield of protoplasts (5.37 x 10^5 per g fwt) was obtained from Enz Sol III in which hemicellulase and sucrose was replaced by cellulose onuzuka R10, mannitol, KCl and $CaCl_2$ (Table III.1). Substitution of cellulose onuzuka R10 with macerozyme in Enz Sol V did not increase the yield but decrease the viability of protoplasts (Table III.1).

Table III.1: Effect of enzyme solutions on protoplasts yield and viability.

Enzyme Solutions	Yield x10^5/g fwt	Viability (%)
Enz Sol I	1.68±0.02	71±1.32
Enz Sol II	3.04±0.18	74.6±1.1
Enz Sol III	5.37±0.26	73.4±2.5
Enz Sol IV	0.48±0.04	96.3±1.23
Enz Sol V	1.84±0.09	64.3±1.65
Enz Sol VI	11.42±0.77	82.3±1.5

The values represent the mean of three independent experiments

A concentration of 0.2 M (3.6 %) mannitol was chosen as the osmotic stabilizer for protoplast isolation due to the fact that the application of sucrose resulted in a tremendous loss of vital protoplasts. This was evidenced by the increased protoplast yields obtained from Enz Sol II and Enz Sol III compared to Enz Sol I consisted of 8 % sucrose (Table III.1). A further increase in protoplasts yields was achieved by adding KCl indicating that KCl plays an important role in cell wall digestion. Subsequently, the suspension callus was digested with various concentration of KCl (0-3 %) of Enz sol III. As shown in Figure III.15, the protoplast yield increased with increasing KCl concentrations to reach a maximum yield at 3 %. The use of KCl concentrations higher than 3 % yielded the highest number of protoplasts, but the digestion mixture then contained extensive aggregation of protoplasts, which would be more favourable for other applications such as protoplast fusion.

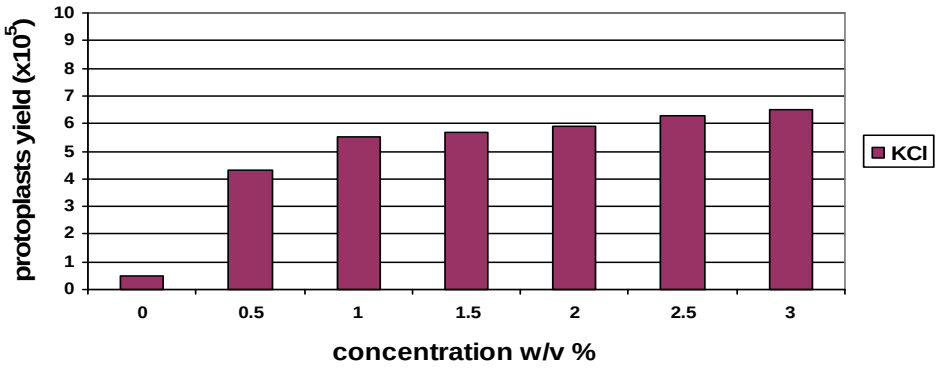

Figure III.15: Effect of KCl concentrations on protoplast yield.

Yields and the vitality of protoplasts were significantly influenced by pectolyase Y23. Hence, the lowest yield and highest viability were obtained when pectolyase Y23 was not presented in Enz Sol IV. Subsequently, the effects of concentration of pectolyase Y23 and incubation time were determined. Various concentrations of pectolyase Y23 (0.05-0.25 %) were added to Enz Sol III, and then was used to digest the suspension callus for 1-24 hours. Release of protoplasts started after one hour with all analyzed concentration of pectolyase Y23 (0.05-0.25 %), and the maximum protoplast yield was obtained with 0.1 % pectolyase Y23 and 14 hours incubation time (Figure III.16).

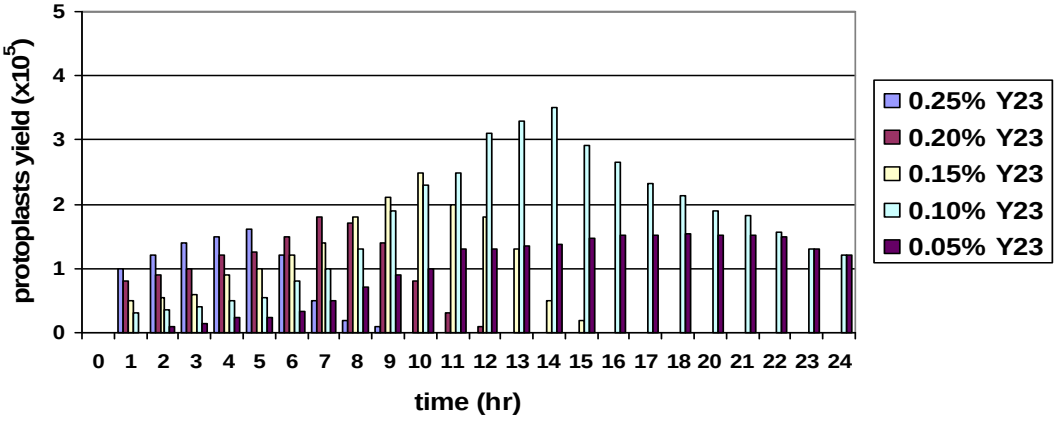

Figure III.16: Effects of Pectolyase Y23 concentrations and incubation time on protoplast yields.

Most of the protocols for protoplast isolation from suspension culture based on gradient or flotation steps. The used of gradient or flotation steps in this study always resulted in low yields ($<10^2$). Subsequently, protoplast purification was performed by centrifugation.

Three washing solutions were adapted from reports by Sambanthamurthi *et al.* (1996) for Wash Sol I, Teulleres and Boudet (1991) for Wash Sol II and Negrutiu *et al.* (1987) for Wash Sol III (Table II.2). Amongst these washing solutions, Wash Sol II was identified as being most efficient because the isolated protoplasts were clean, maintained their viability, and most importantly yielded in sufficient amounts of protoplasts. However, agglutination of protoplasts was frequently observed, which negatively affects the yield. Further studies identified that the amount of $CaCl_2$ in Wash Sol II was crucial for the yield and demonstrated that the highest number of protoplasts were obtained with $CaCl_2$ concentrations up to 0.5% (Figure III.17). In addition, incubation at 26ºC under static condition was identified as the best environment for yielding high amounts of protoplasts. Also incubation on rotary shaker could release the protoplasts in shorter time, cell debris contaminations were a problem.

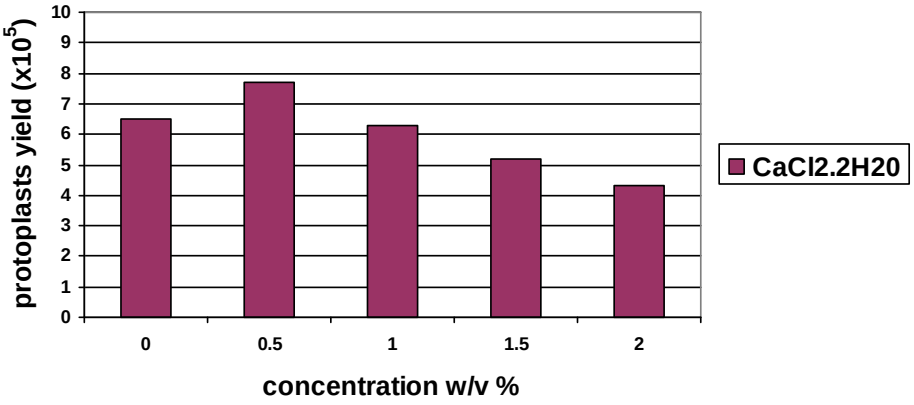

Figure III.17: Effect of $CaCl_2.2H_20$ concentrations on protoplasts yield.

Taken together, the best enzyme and washing solution for efficient protoplast isolation were identified as Enz Sol VI (Table II.1) and Wash Sol IV (Table II.2). Using this combination, protoplasts were successful isolated from different development stage of oil palm embryogenic calli (Figure III.18: A-D). High yield and viability (Table III.1: Enz Sol VI) were obtained for protoplast derived from suspension callus, which are uniform in size (Figure III.18: A) and therefore most suitable to be use for the plant regeneration. Application of this protocol to other tissues such as young leaf, apical explants and roots only resulted in low yield protoplasts (Figure III.18: E-G) demonstrated that the established protocol is unique to oil palm embryogenic calli, in particular for suspension callus.

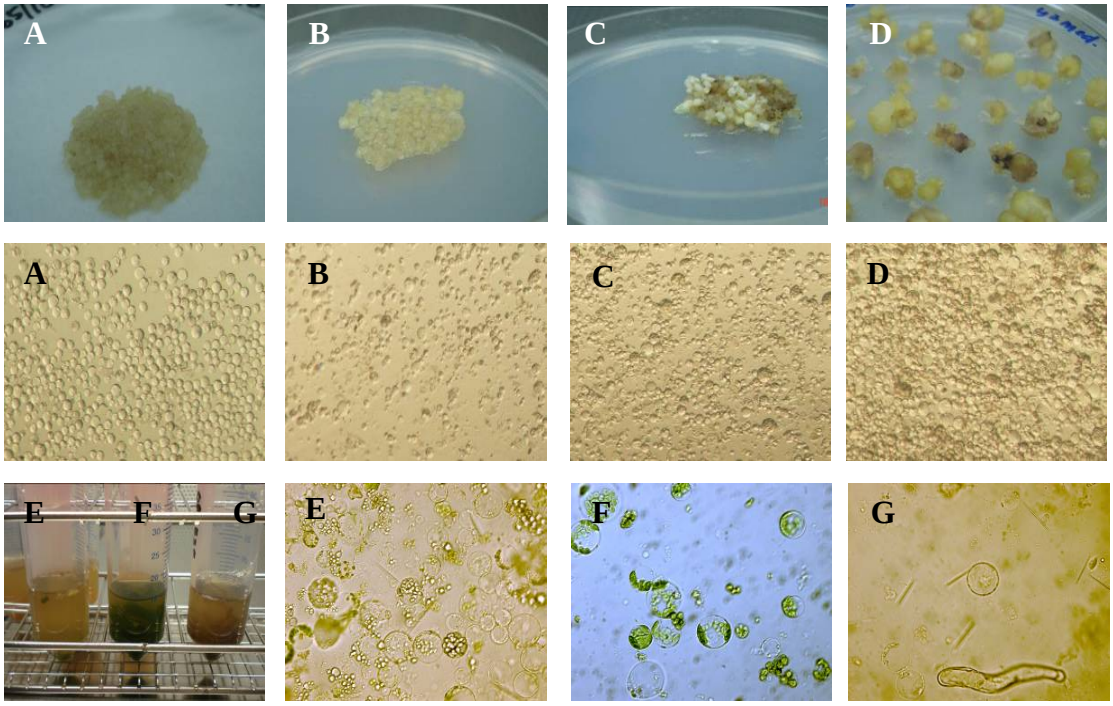

Figure III.18: Freshly isolated protoplasts derived from oil palm tissues.

The oil palm tissues were incubated in Enz Sol VI for 14 hours at 26°C. The protoplasts were purified by washing with Wash Sol IV and then were observed under microscopy as suspension callus (A), yellowish embryos (B), whitish embryos (C), polyembryogenic calli (D), apical explants (E), young leaf (F) and roots (G).

III.3.6 Plant regeneration of oil palm protoplasts

III.3.6.1 Protoplast isolation from oil palm suspension culture

Protoplasts were successfully isolated from suspension callus at 4, 7 and 14 days after subculture of 3 month old suspension culture with yields of 0.9-1.14 x 10^6 per g fwt and with an average viability of 82 %. The yield and viability of the protoplasts obtained from 4 to 14 days after subculture did not show any significant difference. However, the size of protoplasts varied in the range of 5-35 µm. The sizes of the protoplasts were 5-14 µm (Figure III.19: A), 15-25 µm (Figure III.19: B) and 25-35 µm (Figure III.19: C), which were isolated from 4, 7 and 14 days after subculture, respectively. In addition, protoplasts with an average size of 15-25 µm showed a dense cytoplasm concentrated around the nucleus (Figure III.19: D). Meanwhile, 25-35 µm protoplasts were fully packed with cytoplasm (Figure III.19: E), and more than 50 % of the protoplast were less than 15 µm in size and cytoplasm without a nucleus (Figure III:19: F). Subsequently, the protoplasts isolated from 7 days after subculture were used for further experiments.

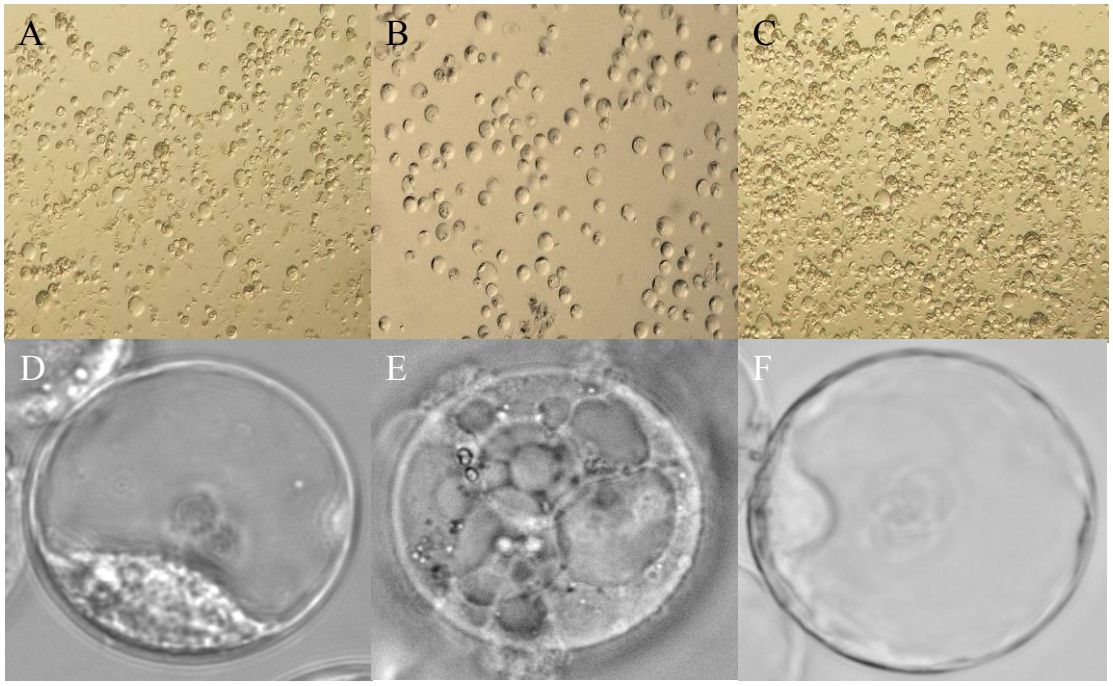

Figure III.19: Physical properties of the protoplasts isolated from different days subculture of oil palm suspension culture.

Freshly isolated protoplasts from suspension callus after subculture at 4 days (A), 7 days (B), 14 days (C). CLSM images showing the cytoplasm structure of protoplast isolated from 7 days (D), 14 days (E) and 4 days (F) of suspension callus.

III.3.6.2 Selection of optimum media

In order to identify appropriate media for cultivation of oil palm protoplasts, 14 media combinations were compared, which was shown in Appendix 1. The components of the media contributed to successful protoplast culture. EC version media (1/2ECI-ECVI) were prepared based on MS basal media (Murashige and Skoog, 1962) except for ECII, which was based on KM basal media (Kao and Michayluk, 1975). Meanwhile, Y3 version media (Y3-Y3F) were based on the modified Y3 basal media (Teixeira *et al.*, 1995). Other components of the media were included based on media used in other plant species.

Preliminary experiments indicated that when protoplasts were cultured in liquid media, division was not observed in all analyzed media and extensive aggregation of protoplasts was observed. Most of the protoplasts died after two week of cultivation. In contrast, protoplasts embedded in agarose solidified remained viabile (Figure III.20: A), showed cell wall formation (Figure III.20: B) and cell division (Figure III.20: C-E) in all tested media.

Overall, Y3-Y3F media was highly superior in its ability to initiate cell wall formation (5-7 days), first cell division (9-14 days) and second cell division (17-21 days; Figure III.20: F) of oil palm protoplasts (Table III.2). The highest protoplast division frequency at 12 % was recorded in Y3A medium with cell wall and first cell division occurring at five days and nine days cultivation, respectively. This follows Y3D medium with 8 % of protoplast division frequency at 10 days, and needs another seven days for development of three cells of protoplasts. EC version media caused a lag time of 10-15 days prior for cell wall formation and the frequency of protoplast division was low (0.5-2.1 %).

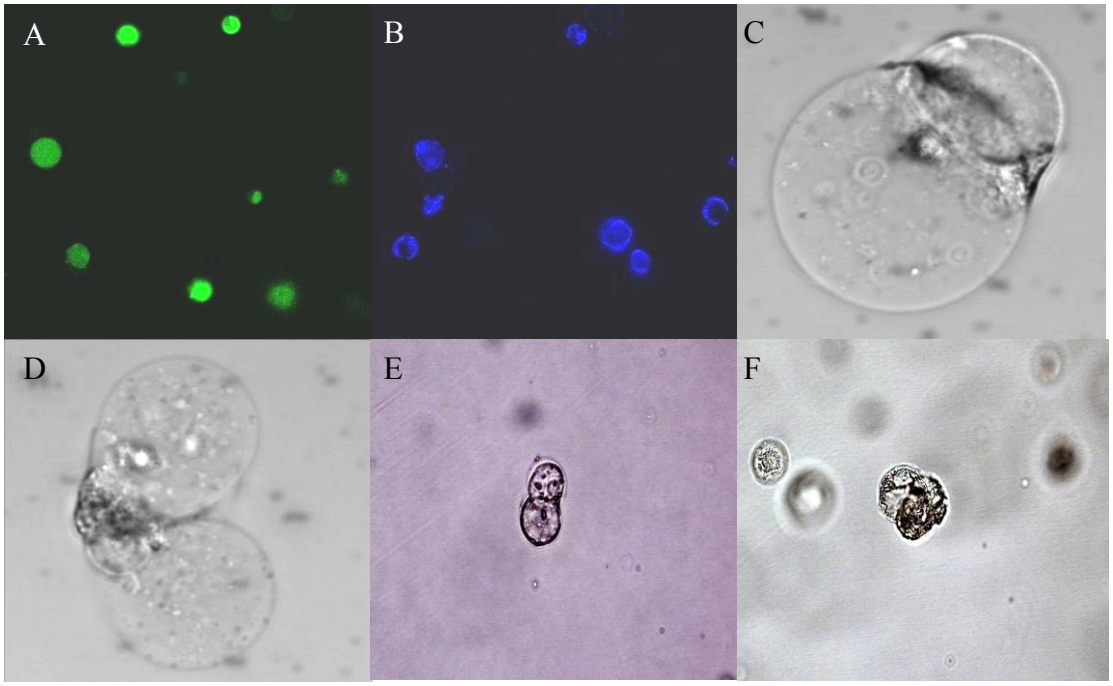

Figure III.20: Development of protoplasts in agarose solidified media.

The protoplasts was cultured in 0.6 % agarose solidified media showing viability after stained with CFDA (A), cell wall formation after stained with fluorescent brightener 28 (B), elongation of cell (C), initial cell division (D), first cell division (E) and second cell division (F).

Table III.2: Protoplast division frequency in different media.

Media	Days for Cell wall formation	Days for 1st division	Days for 2nd division	Division frequency
1/2ECI	15	21	28	0.49±0.23
ECI	15	21	28	0.55±0.19
ECII	10	17	23	1.77±0.19
ECIII	12	17	25	1.10±0.38
ECIV	13	19	24	1.11±0.19
ECV	13	20	26	0.67±0.58
ECVI	10	17	21	2.11±0.19
Y3	6	14	21	3.21±0.5
Y3A	5	9	12	12±0.88
Y3B	7	14	21	4±0.33
Y3C	6	12	19	5.56±0.19
Y3D	5	10	17	7.94±0.58
Y3E	7	14	21	4.89±0.38
Y3F	5	10	17	6.24±0.19

The values of division frequency represent the mean of three replicates.

III.3.6.3 Selection of optimum plant growth regulators (PGRs)

After 8 weeks cultivation of the divided protoplasts, the media became unable to promote the development of microcolony (7-12 cells). The protoplasts were observed to divide into hardly more than five cells and half of them maintained 3-4 dividing cells (Figure III.21: A). Therefore, the addition of plant growth regulators (PGRs) in the used media was found to be essential and had to be optimized to induce the microcolony formation. Eleven combinations of PGRs (Table II.3; PGRs No. 1-11) consisting of different concentrations of four auxins (NAA, 2,4-D, IAA, IBA) and four cytokinins (Zea, GA3, BA, 2iP) were tested in order to regulate the growth of the protoplast cultures in Y3A and Y3D media.

Table III.3 showed that all concentrations of PGRs for combination nos. 1-6 completely inhibited the growth of protoplasts either in Y3A or Y3D media. The concentration of 2,4-D, IBA, GA3 and 2iP higher than 7 µM inhibited cell division, and surprisingly, the protoplasts died within a week. The formation of microcolonies was observed in both media after 12-16 weeks at all concentrations of PGRs for combination nos. 9-11 (Figure III.21: B). The protoplasts cultured in Y3A medium supplemented with the combination PGRs no. 10 at a concentration of 10 µM NAA, 2 µM 2,4-D, 10 µM IAA, 2 µM IBA, 10 µM Zea, 2 µM GA3, 10 µM BA and 2 µM 2iP gave the highest division frequency at 18.33 % and microcolony formation frequency at 8.86 % (Figure III.21: C).

For Y3D medium, the PGRs combination no. 10 was also able to promote 7.38 % of dividing protoplasts to the 3.14 % of microcolony formation in which the division frequency was slightly lower compared to 8.19 % division frequency of Y3D medium without PGRs.

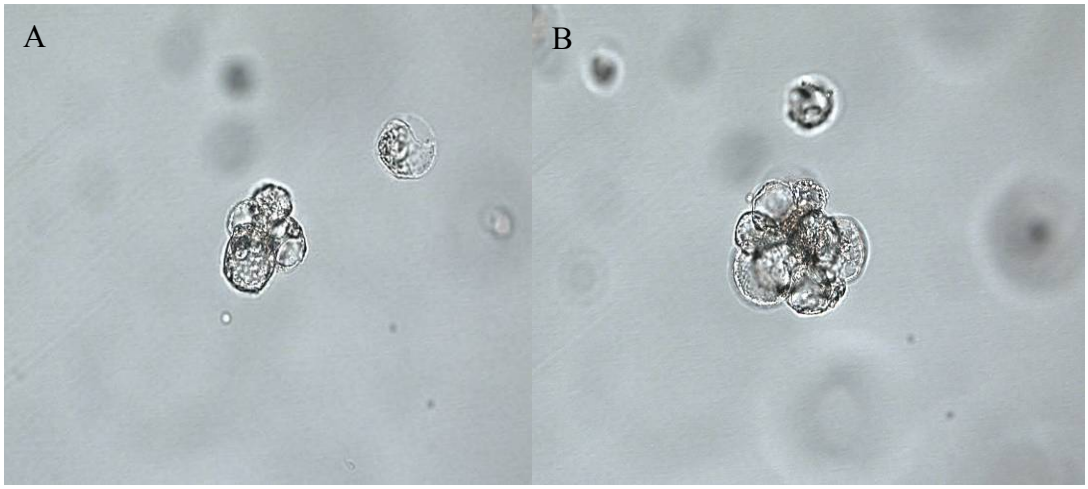

Figure III.21: Microcolony formation in Y3A medium.

Protoplasts maintained in 4 dividing cells in Y3A medium without PGR (A). The microcolony formation in agarose solidified Y3A medium supplemented with PGR combination no. 10 (B).

Table III.3: Effect of PGRs for protoplasts culture using Y3A and Y3D media.

PGRs No.	Agarose solidified culture			
	Y3A		Y3D	
	Division frequency	Microcolony frequency	Division frequency	Microcolony frequency
Without PGR	11.16±0.9	-	8.19±1.34	-
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	3.51±1.09	1.82±0.46	0.53±0.92	
8	6.64±2.24	2.98±0.61	1.76±1.54	-
9	9.25±1.01	4.40±0.78	4.64±1.06	0.73±0.66
10	18.33±0.44	8.86±0.85	7.38±1.16	3.14±0.65
11	14.37±1.83	6.39±0.93	6.29±2.33	1.46±0.62

The values represent the mean of three independent experiments

III.3.6.4 Selection of optimum PGRs in Y3A medium

In order to study the effect of each hormone in the PGRs combination no. 10 on the development of microcolonies into microcalli (15-30 cells), seven combinations of PGRs were evaluated in Y3A medium by excluding one of the hormones in each combination as shown in Table II.3 (PGRs nos. 12-18). Among the analyzed PGRs combinations, the numbers of microcolonies significantly increased at frequencies of 11.3 %, 11.9 % and 13 %, when cultured with PGRs combination nos. 12, 13 and 15 in which 2 µM 2,4-D, 10 µM IAA and 10 µM Zea were excluded (Table III.3). In contrast, when excluding the concentration 2 µM of each IBA, GA3 and 2iP in PGRs combination no. 14 and 16-18, the microcolony frequencies decreased to a range between 3.56 % and 5.03 %. The microcolonies developed further into microcalli and were clearly visible by eye (Figure III.22) after 20-24 weeks cultures in Y3A medium with PGRs combinations nos. 12, 13, 15 and 17, in which the highest microcalli formation frequency of 6.13 % was obtained from PGRs combination no. 15 and the lowest of 1.6 % from the combination no. 12. The absence of IBA, GA3 and 2iP in PGRs combinations nos. 14, 16 and 18 adversely influenced the formation of microcolonies and no microcalli could be developed even when culturing time was extended to 26 weeks.

Table III.4: Effect of PGRs for protoplasts culture using Y3A medium.

PGRs No.	Agarose solidified culture	
	Y3A	
	Microcolony frequency	Microcalli frequency
10 (control)	7.76±0.38	0
12	11.3±2.42	1.6±0.7
13	11.9±1.55	5.5±2.13
14	3.56±2.64	0
15	13.06±2.95	6.13±1.58
16	6.6±0.96	0
17	5.03±1.95	2.73±2.12
18	4.53±1.65	0
	Agarose beads culture	
	Microcolony frequency	Microcalli frequency
19	22.2±4.75	9.8±1.87

The values represent the mean of three independent experiments

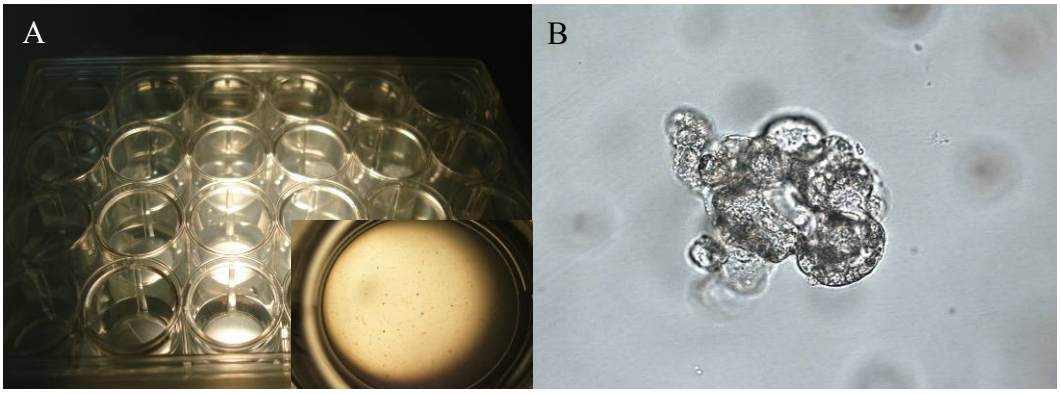

Figure III.22: Microcalli formation in Y3A medium.

Microcalli in 24 wells plate were visible after 24 weeks cultivation (A; Small picture is magnification of individual well). The microcalli formation in agarose solidified Y3A medium supplemented with PGR combination no. 15 (B).

III.3.6.5 Effects of osmotic pressure, optimum PGRs using agarose beads culture

Based on the results of PGR optimization, the protoplasts were cultured using the agarose beads technique (Figure III.23) comprising Y3A medium supplemented with 10 µM NAA, 2 µM 2,4-D, 2 µM IBA, 2 µM GA3 and 2 µM 2iP, which was designated as PGRs combination nos. 19 (Table 3). The agarose beads were cultured for three days by surrounding the beads with 21 % osmotic solution of either sucrose, glucose or mannitol to maintain the osmotic pressure and to prevent the agarose beads from drying out. The use of different types of carbohydrate as the osmotic solution did not adversely effect the protoplast cultures. However, the protoplasts cultured in the osmotic solution were observed to retain a sphere shape and viability compared to those cultured in the absence of osmotic solution where the protoplasts became oval shaped and half of them burst and died. Higher than 21 % osmotic solution quickly changed the agarose beads to a brown color and lead to the formation of pyramid likes crystal on the surface of the agarose beads.

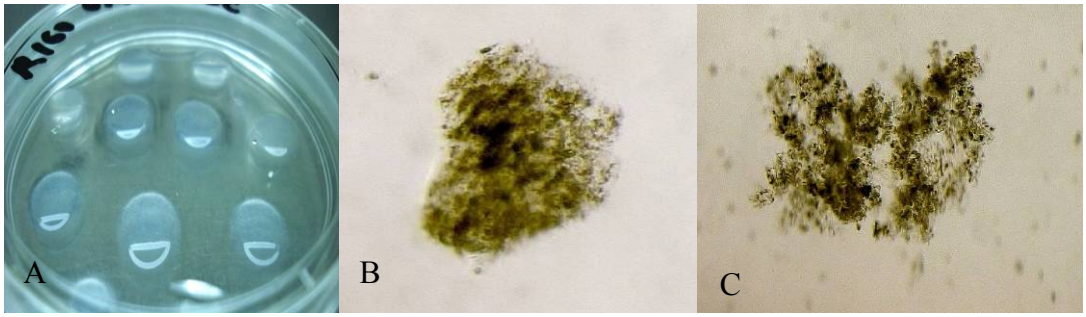

Figure III.23: Protoplasts culture using agarose bead technique.

The protoplasts were embedded in 0.6 % agarose comprising Y3A medium supplemented with PGR combination no. 19 and dropped as beads in petri dish. Then, 21 % v/w sucrose was added as osmotic solution and cultured for 3 days (A). The formation of compact embryogenic (B) and friable (C) callus in agarose bead surrounding with Y35N5D2iP liquid medium consisted of 200 mg/l AA.

After three days, the osmotic solution was replaced with Y3A liquid medium. Longer than five days in the osmotic solution resulted in protoplasts becoming a dark color and they developed fur-like structures on the surface of cell wall which retarded cell division. Culturing the agarose beads in Y3A liquid medium promoted the cell division and development of microcolonies in 12-16 weeks at the frequency of 22.2 % (Table III.4). The microcolony frequency in agarose beads was significantly higher than PGRs combination no. 15 where the frequency was 13 %, which was cultured in agarose solidified medium. At this stage, the agarose beads were cultured in Y3 liquid medium consisting of 4.5 % (w/v) sucrose and resulted in the development of microcalli at the frequency of 9.8 % at compared to 6.13 % of PGRs combination no. 15. Besides using PGRs combination no. 19, the osmotic pressure surrounding the agarose beads was gradually reduced and subsequently increased the numbers of microcolonies and microcalli by two fold.

III.3.6.6 Control of callus browning

After 28 weeks, the microcalli failed to further grow to embryogenic calli. It was observed that the microcalli turned brown and light-dark most probably due to the accumulation of phenolic compounds released from the cells and also the chemical reduction of PGRs in the agarose beads. Adding ascorbic acid (AA), silver nitrate (AgNO3) or activated charcoal (AC) with PGRs to the surrounding media of agarose beads reduced the microcalli browning process and promoted embryogenesis. Thus, the agarose beads were cultured in Y35N5D2iP liquid medium with the addition of different concentrations of AA, AgNO3 and AC. After 4

weeks of cultivation the microcalli become yellowish and then developed embryogenic callus, indicating a further growth of the cells, especially when cultured in media containing 200 mg/l of AA. In comparison, culturing the agarose beads in Y3 liquid media with 200 mg/l AA without PGRs resulted in fewer embryogenic calli forming.

III.3.6.7 Plant regeneration

Eight weeks after culture initiation in Y35N5D2iP liquid medium with the addition of 200 mg/l AA, two types of embryogenic callus developed as compact embryogenic (CE) callus (Figure III.23: B) and friable embryogenic (FE) callus (Figure III.23: C) with some of the embryogenic callus developing out from the agarose beads (Figure III.24: A-B). At this time, the agarose beads were transferred onto Y3 solid medium supplemented with different concentration of PGRs (NAA and BA) to promote the embryogenic calli to enter the somatic embryogenesis stage. Of five different concentrations of PGRs (NAA and BA) tested, only Y3 solid medium supplemented with 1 µM NAA and 0.1 µM BA (Y31N0.1BA) was able to induce the FE embryogenic calli to develop into somatic embryos (Figure III.24: C). In contrast, the CE callus was observed during the development of FE callus prior to the somatic embryogenesis stage. The agarose beads were subcultured in four-week intervals on Y31N0.1BA solid medium until all the embryogenic calli were developed to somatic embryos. After 44 weeks of agarose bead culture, whitenish embryoids (Figure III.24: D) appeared on the surface of agarose beads which were transferred onto ECI solid medium with 1 µM NAA and 0.1 µM BA (ECI1N0.1BA). The greenish embryoids (Figure III.24: F) were observed within eight weeks when cultured in the presence of light and regenerated into plantlets in another 12 weeks (Figure III.24: G-H).

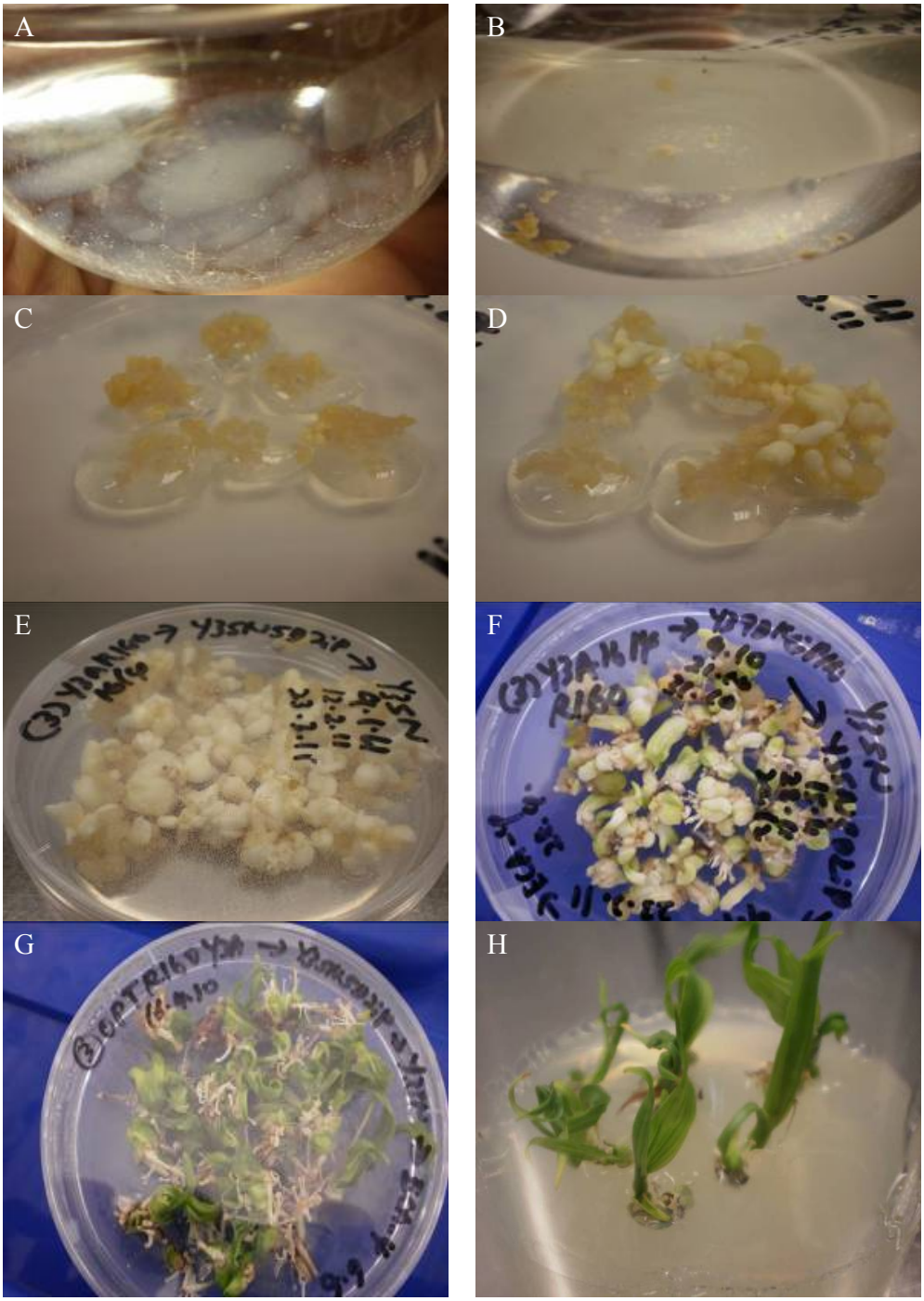

Figure III.24: Plant regeneration from embryogenic calli derived from protoplasts.

Agarose beads containing microcalli (A) derived from protoplasts were cultured in Y35N5D2iP liquid medium supplemented with 200 mg/l ascorbic acid for development of embryogenic calli (B) within 9 months. Then, the agarose beads were cultured on Y31N0.1BA medium (C) for 2 month to generate embryos (D). After 2 months cultured on ECI1N0.1BA medium (E), the whitenish embryoids were growth to the greenish embryoids (F) and then to shoots (G) and plantlets (H) within 3 to 5 months.

III.3.7 PEG-mediated transient expression of oil palm protoplasts

III.3.7.1 Effect of protoplasts from different ages of suspension cultures

In order to identify the most suitable protoplasts for DNA uptake using PEG-mediated transformation, different sources of protoplasts were used defined as 7 days and 14 days after subculture of 3 month old suspension culture, or 4 month old suspension culture. Initial protoplast transfection experiments used 10 μg of CFDV-hrGFP plasmid DNA, incubation for 10 minutes and mixing with 40 % (w/v) PEG dissolved in Rinse solution. The presence of GFP-fluorescent protoplasts indicated expression of the hrGFP gene and these were detected at 72 hours after PEG-mediated transformation for all sources of protoplasts (Figure III.25). However, only low transfection efficiencies (<0.1 %) were achieved in which GFP fluorescence was only observed in viable (i.e. not ruptured) protoplasts. Protoplasts isolated from 7 and 14 days subcultures showed GFP fluorescence localized throughout the cytoplasm and nucleus extending to the plasma membrane (Figures III.25: A and B), whilst, GFP fluorescence was distributed throughout the whole cell for protoplasts from 4 month old suspension culture (Figure III.25: C). In addition, low and high intensity pale yellow autofluorescences were detected in protoplasts from 14 day subculture of 3 month and 4 month old suspension cultures, respectively, which is shows in the merged images (Figures III.25: B and C). Thus, the protoplasts isolated from 7 day subculture of 3 month old suspension culture were the most suitable for PEG-mediated transformation due to no autofluorescences which would result a false GFP positive.

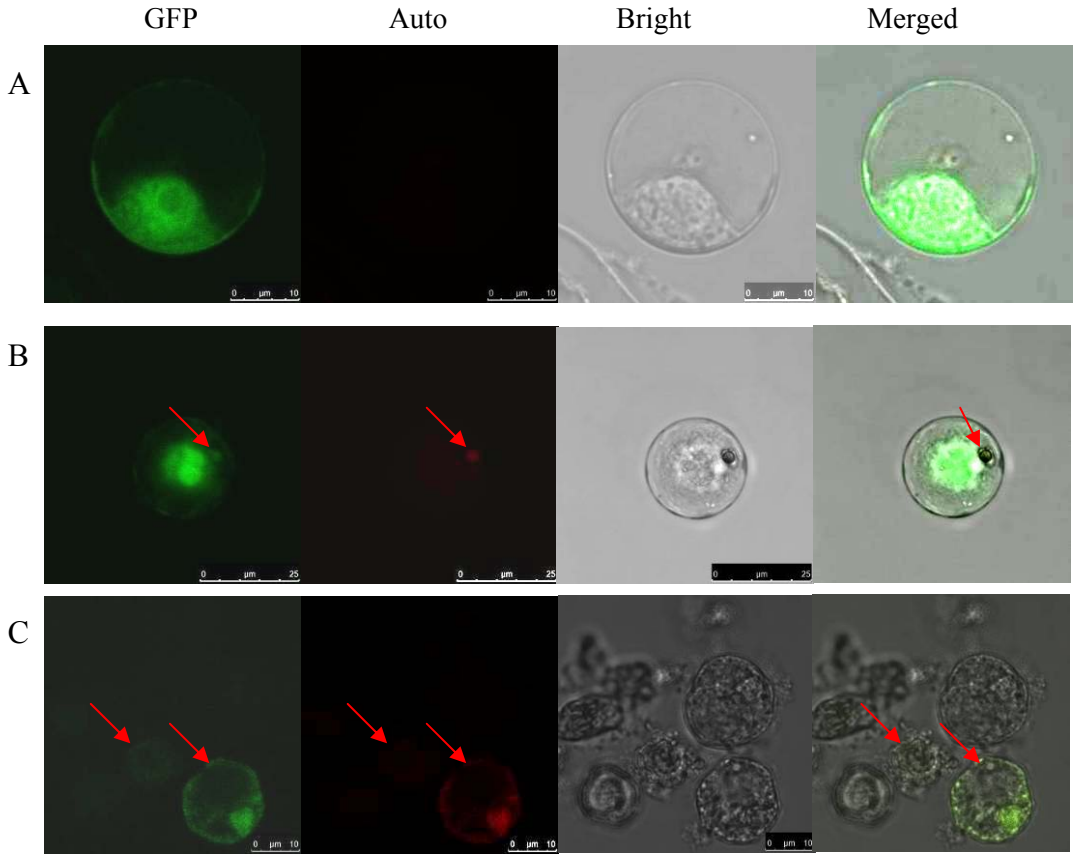

Figure III.25: GFP fluorescence in oil palm protoplasts.

Transient GFP fluorescence in protoplasts isolated from 3 month old suspension culture after subculture at 7 days (A) and 14 days (B), and protoplasts isolated from 4 months old suspension culture (C). CLSM images of GFP fluorescence (GFP), autofluorescence (Auto), bright field (Bright) as well as three-layer images (Merged) of protoplasts are shown. Red arrows indicate the autofluorescence of protoplasts.

III.3.7.2 Effect of $MgCl_2$ on transfection efficiency

In order to examine the effects of Mg^{2+} ions on transfection efficiency, oil palm protoplasts were incubated for 10 minutes with 10 µg of CFDV-hrGFP plasmid DNA and mixed with 40 % (w/v) PEG dissolved in Rinse solution comprising 10 mM, 25 mM, 50 mM and 100 mM of $MgCl_2.6H_20$. Addition of 10 mM $MgCl_2.6H_20$ in PEG solution resulted in drastically increased the transfection efficiency by 4 folds (0.43 %, Figure III.26: A) compared to a solution without $MgCl_2.6H_20$ (<0.1 %). The transfection efficiencies were consistently increased to 2.43 % by the increasing of $MgCl_2.6H_20$ concentrations (Figure III.26: B-D). Furthermore, the expression of the hrGFP gene was highly and consistently influenced by the presence of Mg^{2+} ions as indicated from low to high intensity of GFP fluorescence (Figure III.26: A-D).

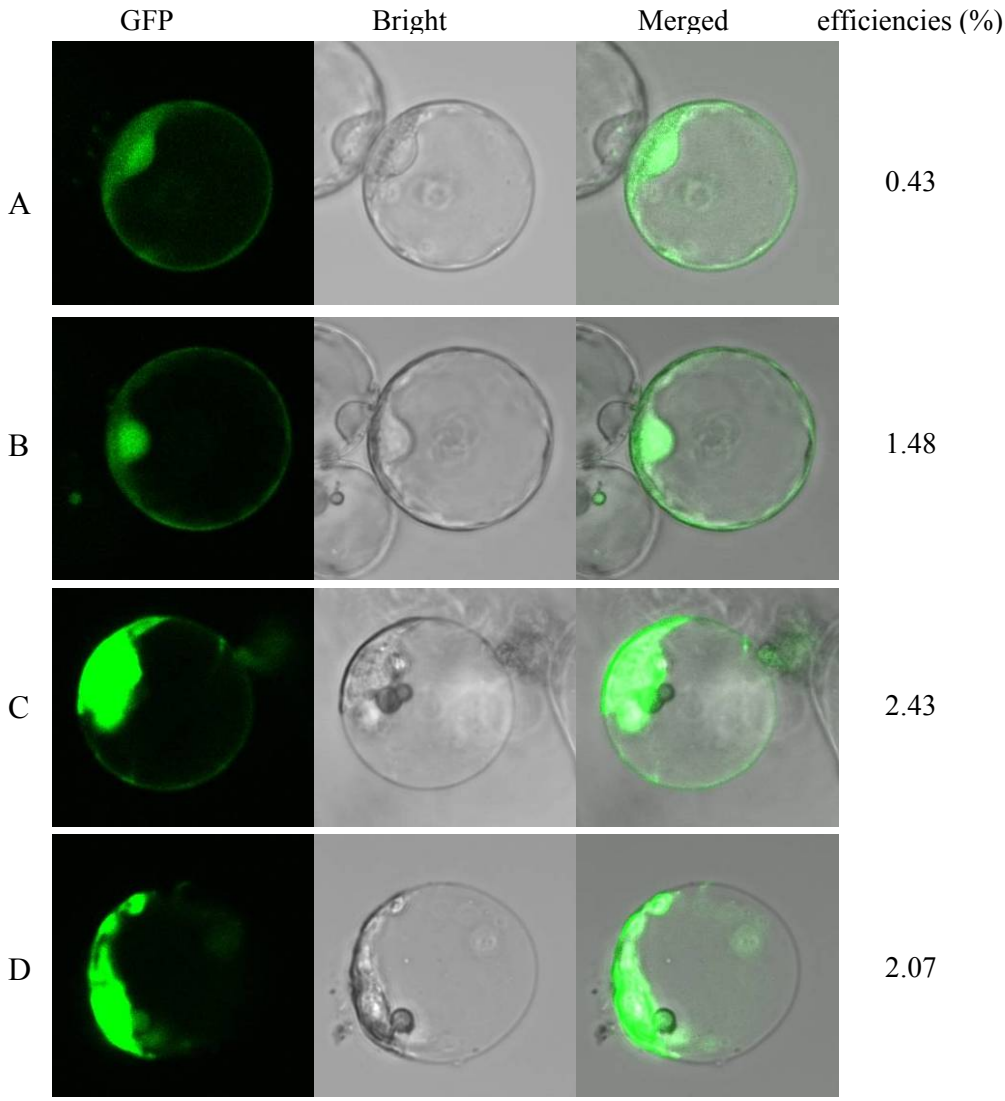

Figure III.26: Transfection efficiency affected by concentrations of $MgCl_2.6H_20$.

Oil palm protoplasts were transfected with 10 ug CFDV-hrGFP plasmid using 40 % (w/v) PEG solution consisted with $MgCl_2.6H_20$ of 10 mM (A), 25 mM (B), 50 mM (C) or 100 mM (D). Transfection efficiency was calculated as the percentage of the number of GFP-fluorescent protoplasts divided by the total number of protoplasts in one representative microscope field.

III.3.7.3 Effect of DNA incubation and carrier DNA on transfection efficiency

The incubation period after the addition of plasmid DNA to the protoplasts was extended to 30 minutes to increase transfection efficiency. However, when DNA incubation was prolonged to 15 or 30 minutes before addition of PEG-MgCl solution, the transfection efficiency was rapidly decreased to 1.34 % and further to 0.75 % (Figure III.27: A and B). Thus, this result suggesting the PEG-MgCl solution should be added immediately at 10 minutes or less to avoid the decreasing of transfection efficiency. Meanwhile, addition of carrier DNA in the form of 50 µg of sonicated salmon sperm DNA mixed with 10 µg of

CFDV-hrGFP plasmid DNA and then incubated for 30 minutes reduced transfection efficiency to 0.69 % (Figure III.27: C).

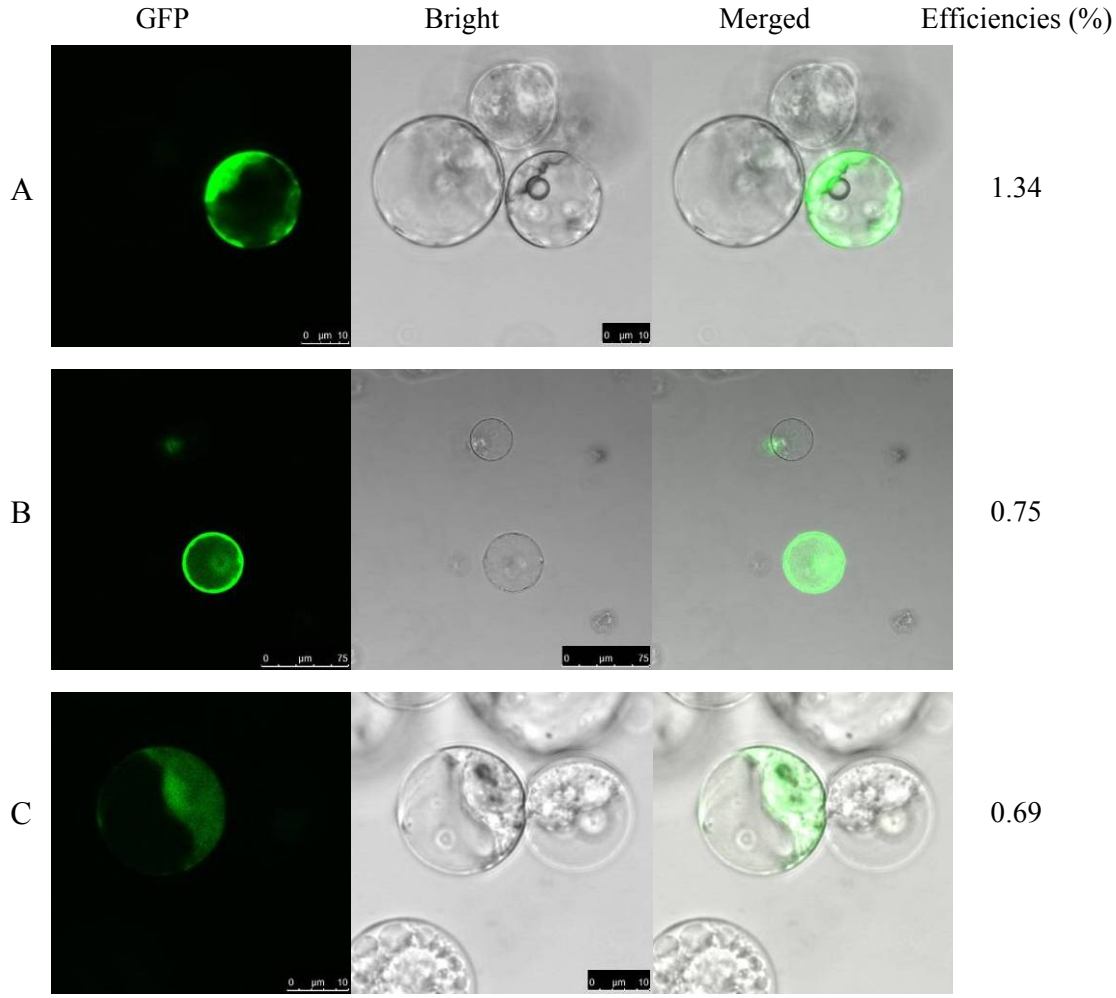

Figure III.27: Effects of DNA incubation and carrier DNA on transfection efficiency.

Oil palm protoplasts were incubated with 10 ug CFDV-hrGFP plasmid DNA for 15 min (A) or 30 min (B), and then mixed with PEG-MgCl solution. Fifty ug carrier DNA also mixed with 10 ug CFDV-hrGFP plasmid DNA and then incubated with the oil palm protoplasts for 30 min (C).

III.3.7.4 Effect of DNA concentration on transfection efficiency

To investigate the effects of the amount of DNA introduced into oil palm protoplasts on transfection efficiency, 25 μg and 50 μg of CFDV-hrGFP plasmid DNA was transfected into oil palm protoplasts by using PEG-MgCl solution (40 % (w/v) PEG and 50 mM $MgCl_2.6H_20$). The results showed transfection efficiencies of 1.8 % for 25 μg which increased to 2.42 % for 50 μg DNA (Figure III.28: A and B). Based on the intensity of GFP fluorescence, high level of hrGFP gene expression was observed at both concentrations of plasmid DNA. GFP fluorescence concentrated in the cytoplasm for 25 μg of plasmid DNA and GFP fluorescence distributed over the whole cell of protoplasts for 50 μg of plasmid DNA. Subsequently, the optimal concentration 50 μg of DNA provides the greatest transformation efficacy in PEG-mediated transformation.

III.3.7.5 Effect of PEG concentration on transfection efficiency

PEG at a molecular weight of 4000 was selected to optimize the effect of PEG concentration on transfection efficiency of oil palm protoplasts. PEG concentrations at 25 % (w/v), 40 % (w/v) and 50 % (w/v) were used to transfect 50 μg of CFDV-hrGFP plasmid DNA into oil palm protoplasts which resulted in the transfection efficiencies of 3.56 %, 2.42 % and 1.94 %, respectively (Figure III.28: C-E). The data show that 25 % (w/v) PEG concentration was the optimal concentration for PEG-mediated transformation of oil palm protoplasts. The intensity of GFP fluorescence was at the same level for all concentration of PEG indicating that hrGFP gene expression was not influenced by PEG concentration. The toxicity of PEG caused the viability of the oil palm protoplasts to reduce to 30-50 % when higher than 25 % (w/v) PEG concentration was used. The damaged protoplasts were observed surrounding the GFP fluorescing (viable) protoplasts which indicated that the oil palm protoplasts were very sensitive to the toxicity of PEG. The GFP-fluorescent damaged protoplasts were also be observed when 40 % (w/v) or 50 % (w/v) PEG concentration was used indicating higher transfection efficiency could be achieved if oil palm protoplasts could withstand the toxicity of PEG.

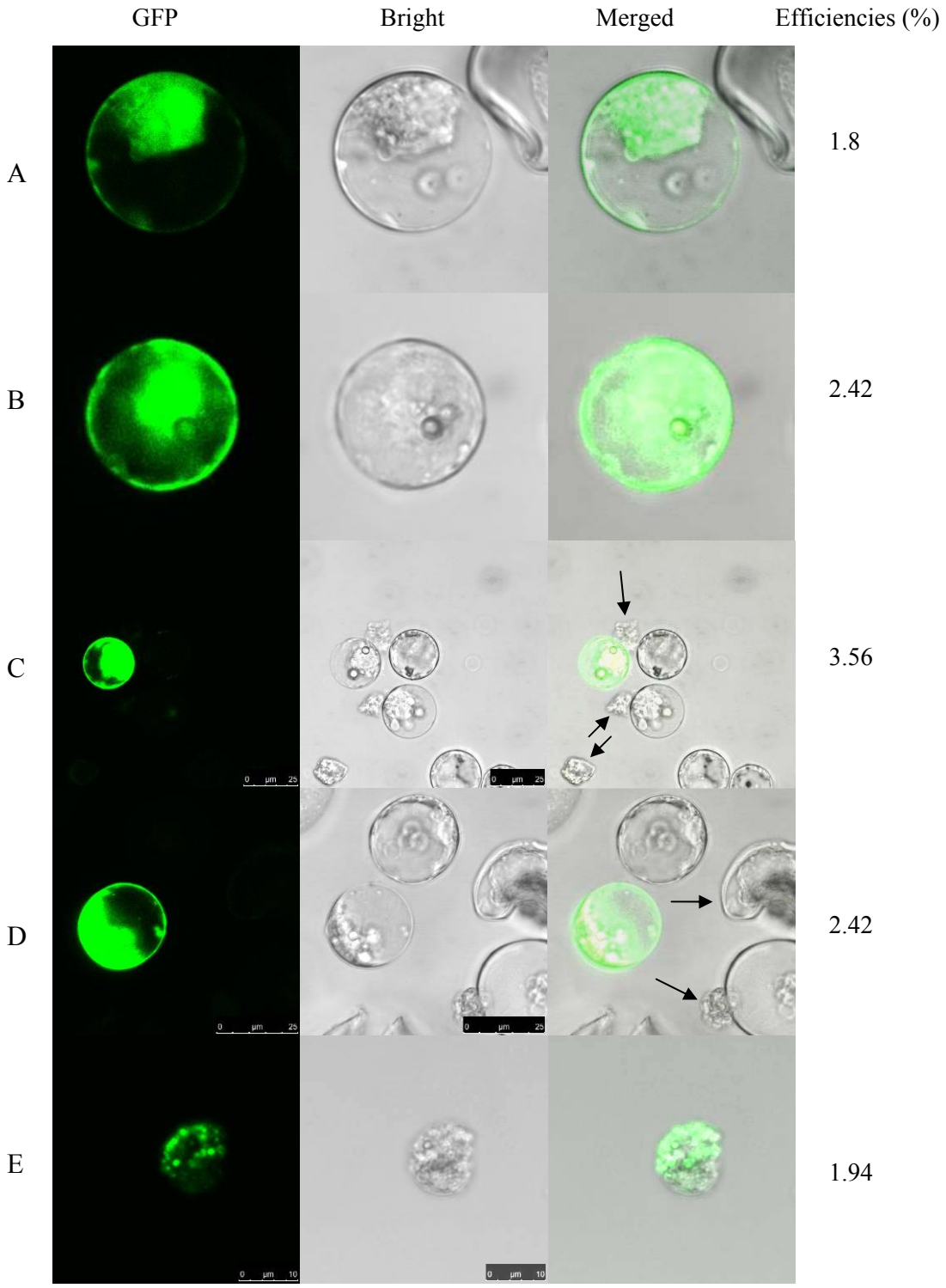

Figure III.28: Effects of DNA and PEG concentrations on transfection efficiencies.

Twenty five ug (A) or 50 ug (B) of CFDV-hrGFP plasmid DNA was transfected into oil palm protoplasts and various concentrations of PEG, 25% (C), 40% (D) or 50% (E), also used to transfect 50 ug of CFDV-hrGFP plasmid DNA into oil palm protoplasts. Black arrows indicate the damaged protoplasts caused by the toxicity of PEG.

III.3.7.6 Effect of heat shock treatment on transfection efficiency

The effect of heat shock treatment was tested using the above optimized protocol. The protoplasts was incubated at 45ºC for 5 minutes and then placed on ice for 1 minute followed by 10 minutes incubation with 50 µg of CFDV-hrGFP plasmid DNA, and then mixed with 25 % (w/v) PEG solution consisting 50 mM $MgCl_2.2H_20$. The transfection efficiency was further increased to 4.22 % when heat shock treatment was incorporated with the optimized protocol (Figures III.29: A). The GFP- fluorescent protoplasts were observed continuously for 9 days indicated that hrGFP gene expression retained with a less decrease in transfection efficiency, 4.08 % at days 6 and 3.93 % at days 9, without being interfered by the Washing solution (Figures III.29: B and C).

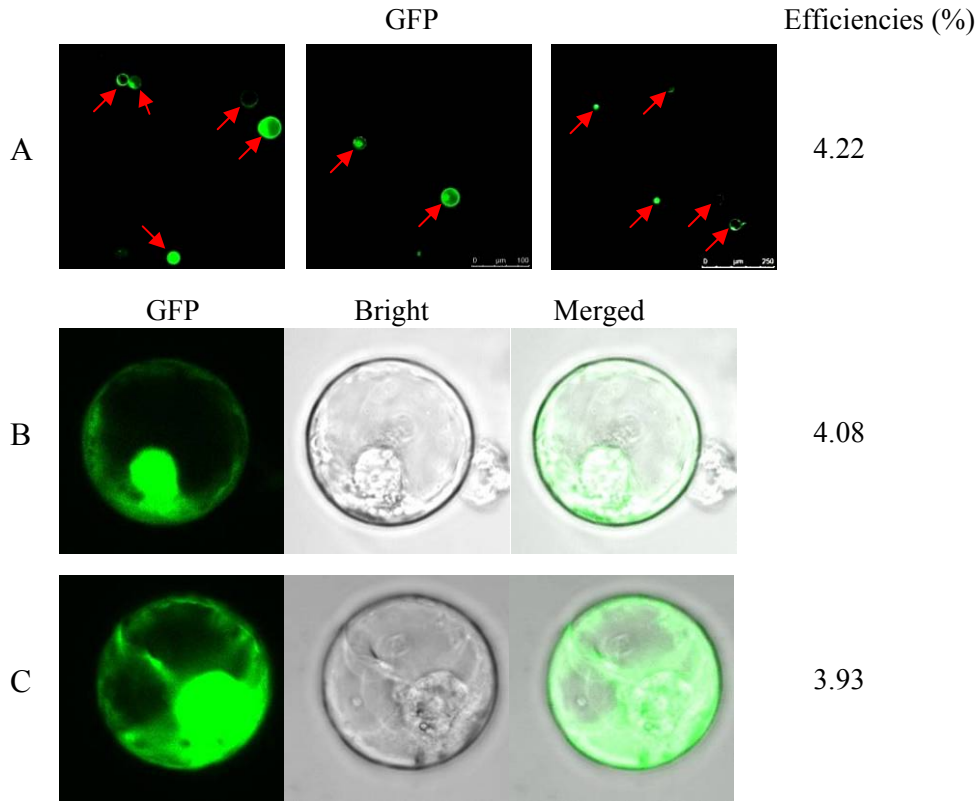

Figure III.29: Effect of heat shock treatment on transfection efficiency.

The protoplasts were incubated at 45ºC for 5 minutes and then on ice for 1 minute before mixed with 50 ug of CFDV-hrGFP plasmid DNA, which was transfected into oil palm protoplasts by using PEG-MgCl solution (40% PEG, 50 mM $MgCl_2.6H_20$, 3% KCl and 3.6% mannitol, pH 6.0), and then incubated at 26ºC for 3 days (A), 6 days (B) and 9 days (C). Red arrows indicate the GFP-fluorescent protoplast.

III.3.8 Stable transformation of oil palm protoplasts mediated by DNA microinjection

III.3.8.1 Choice of protoplast platform

The alginate layer embedded-oil palm protoplasts (Figure III.30: A) were used for DNA microinjection since the protoplasts are in a single planar position (Figure III.30: B). Various concentrations of alginate (0.5-2 %) were dissolved in Y3A liquid medium (5.5 % sucrose and 11.9 % glucose supplemented with 10 µM NAA, 2 µM 2,4-D, 2 µM IBA, 2 µM GA3, 2 µM 2iP and 200 mg/l ascorbic acid) and used to embed the oil palm protoplasts for DNA microinjection. As a result, 1 % alginate was the optimal concentration to fix the protoplasts in one plane which made it easier to facilitate injection. In contrast, lower and higher than 1 % alginate resulted in the moveable and accumulation of protoplast clumps, respectively.

III.3.8.2 Optimal time for DNA microinjection

Alginate layer-embedded protoplasts were cultured for 3-4 days in a two compartment dish (Figure III.30: C) for partial development of the cell wall which was an optimal time for DNA microinjection. Freshly embedded protoplasts were damaged when the needle tip touched the plasma membrane demonstrating that the fragile membrane alone is sufficiently not hard enough to withstand the penetration of the needle tip. Meanwhile, DNA microinjection using protoplasts after 5 days of culture were difficult due to the cell wall being fully developed. Only one micromanipulator was used to inject the protoplasts because the protoplasts were firmly fixed inside the alginate layer (Figure III.30: D).

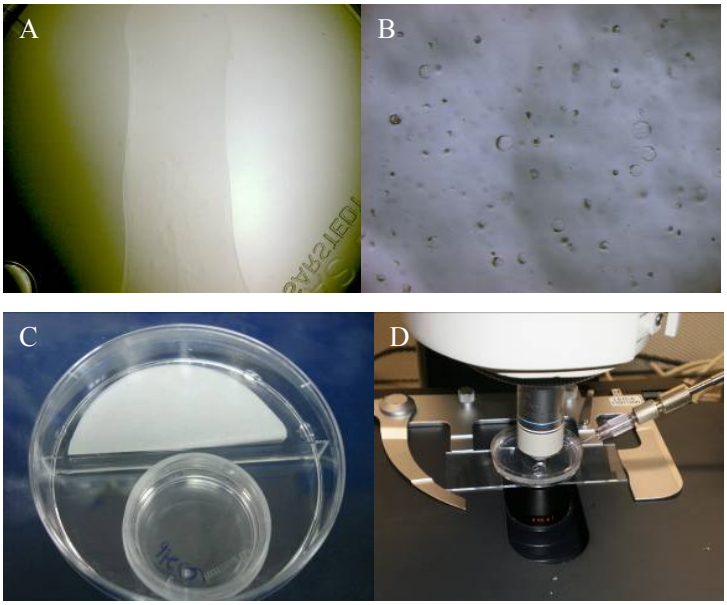

Figure III.30: DNA microinjection of oil palm protoplasts.

The protoplasts were isolated from 7 days after subculture of 3 months old suspension culture and mixed with 1% alginate solution consisted of Y3A medium and then distributed as a thin layer onto supporting medium (A). The embedding protoplasts were in single planar layer confirmed by using the 10x objective (B). The dish was placed in two compartment dish and incubated at 28ºC in the dark for 3 days (C), and then placed on the microscope stage for DNA microinjection (D).

III.3.8.3 Effect of Lucifer yellow dye and protoplast compartments on DNA microinjection

Lucifer Yellow dye was essential as guidance for monitoring the DNA injection solution inside the target compartment of oil palm protoplasts (Figure III.31: A and B). Two compartments, nucleus (Figure III.31: C and D) and cytoplasm (Figure III.31: E and F), were successfully injected using a DNA fragment of CFDV-hrGFP. The expression of hrGFP gene was only detected at 72 hours after DNA microinjection, which GFP fluorescence was localized throughout the cytoplasm and nucleus extending to the plasma membrane. No green fluorescence was detected in the protoplasts injected with only Lucifer Yellow dye demonstrating the fluorescent protoplasts were from the expression of hrGFP gene. It was found that the Lucifer Yellow dye could maintain the fluorescence property for only 48 hours at 28ºC.

hrGFP gene expression in the protoplast by nucleus DNA microinjection was detected up to 9 days (Figure III.31: G and H) and disappeared at day 14 demonstrating that the protoplast was damaged and subsequently died after several days of cultivation. In contrast, the

cytoplasmic DNA microinjection resulted in the increased volume of cytoplasm expressing the hrGFP gene at day 9 as shown in Figure III.31 (I and J). Initial cell division was observed after 12 days (Figure III.31: K and L) and divided to 2 and 3 cells at days 21-31 (Figure III.31: M and N), and then further developed to 4-6 cells within 4-6 weeks (Figure III.31: O and P). Fifty to 100 1 % alginate embedded-protoplasts were successfully injected within an hour by cytoplasmic DNA microinjection. Subsequently, the cytoplasm was used as the target compartment for DNA microinjection in further experiments.

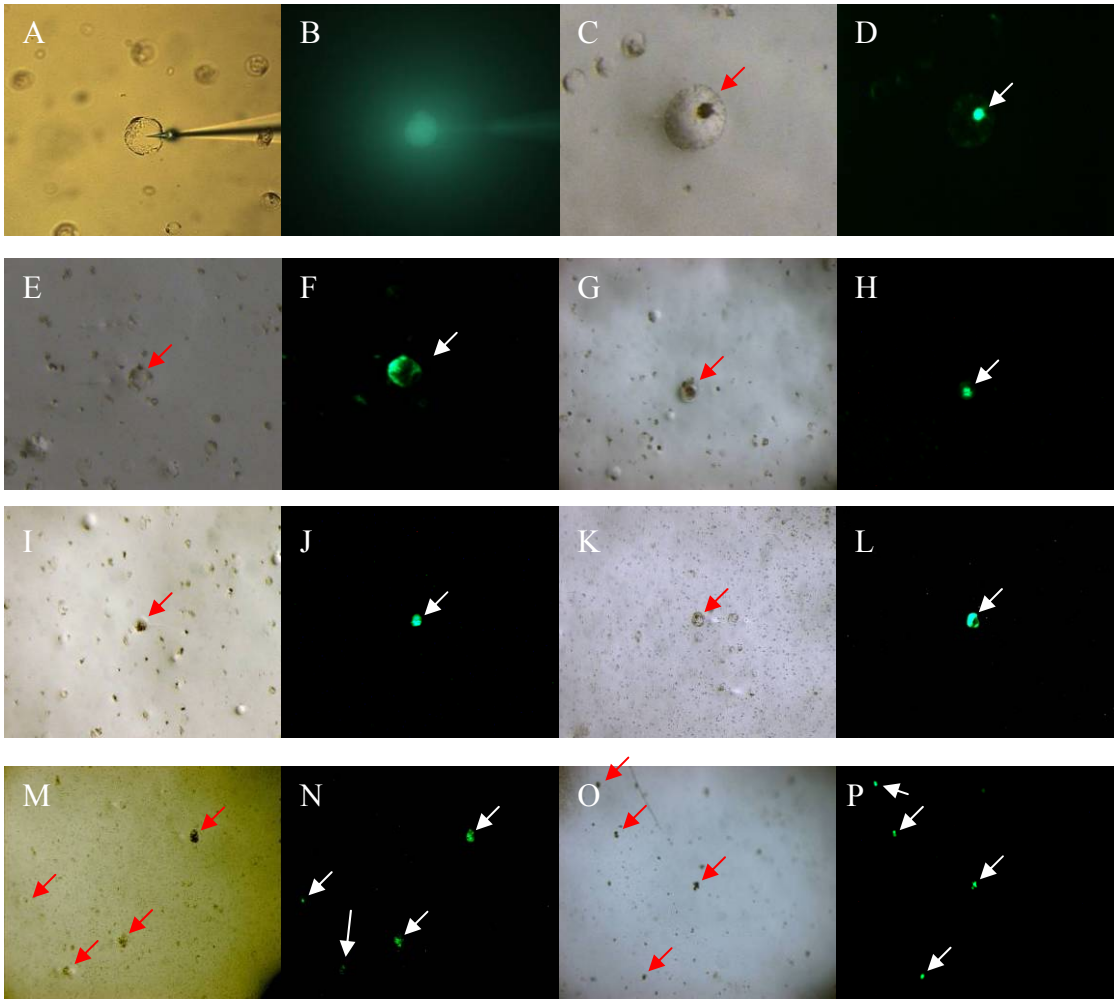

Figure III.31: DNA microinjection into the nucleus and cytoplasm of oil palm protoplasts.

The DNA injection solution was injected into the protoplast (A) and the injection was confirmed by the fluorescence illumination (B). The GFP-fluorescent protoplast was detected after 3 days of nucleus (C and D) and cytoplasmic (E and F) DNA microinjections. The GFP-fluorescent protoplast was observed at day 9 (G and H) after nucleus DNA microinjection. The GFP fluorescence in the protoplasts from cytoplasmic DNA microinjection after 9 days (I and J), 12 days (K and L), 21-31 days (M and N) and 4-6 weeks (O and P) of cultivation. Arrows indicate the injected protoplasts.

III.3.8.4 Effect of DNA concentration on transformation efficiency

The optimal DNA fragment concentration was determined by DNA microinjection with three different concentrations, 100 ng/µl, 500 ng/µl and 1000 ng/µl of DNA injection solution. Fifty cells in the alginate layer-embedded protoplasts were injected with each concentration of DNA. After a month, 78 % (39/50), 40 % (20/50) and 10 % (5/50) of transformation efficiencies were obtained from the protoplasts injected with 100 ng/ul (Figure III.32: A), 500 ng/µl (Figure III.32: B) and 1000 ng/µl (Figure III.32: C), respectively.

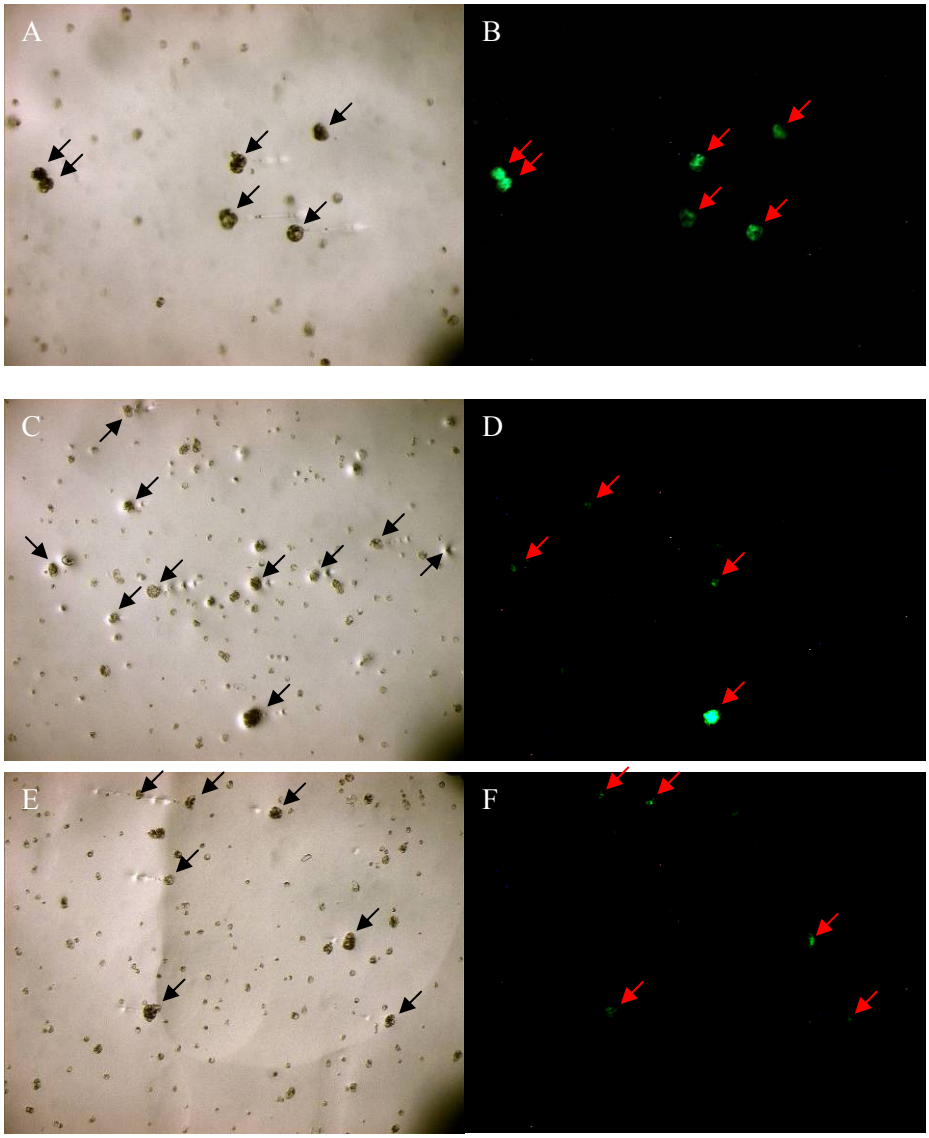

Figure III.32: Effect of DNA concentration on transformation efficiency.

GFP-fluorescent protoplasts after a month cultured which were injected with 100 ng/µl (A and B), 500 ng/µl (C and D) and 1000 ng/µl (E and F) of DNA solution. Arrows indicate the injected protoplasts.

III.3.8.5 Development of microcalli expressing GFP gene

The development of microcolonies which were injected with the optimal DNA concentration (100 ng/µl) were observed in 2-3 months but the transformation efficiency was decreased to 34 % (17/50) (Figure III.33: A and B). The microcolonies maintained the expression hrGFP gene for another 2 months (Figure III.33: C and D) and decreased to 10 % of transformation efficiency (5/50) when the microcalli were developed in 6 months (Figure III.33: E and F). The microcalli expressing hrGFP were removed from the alginate layer (Figure III.33: G) and were transferred to Y31N0.1BA solid medium (Figure III.33: H) for further development of embryogenic calli which was similar to plant regeneration from protoplasts using agarose beads culture.

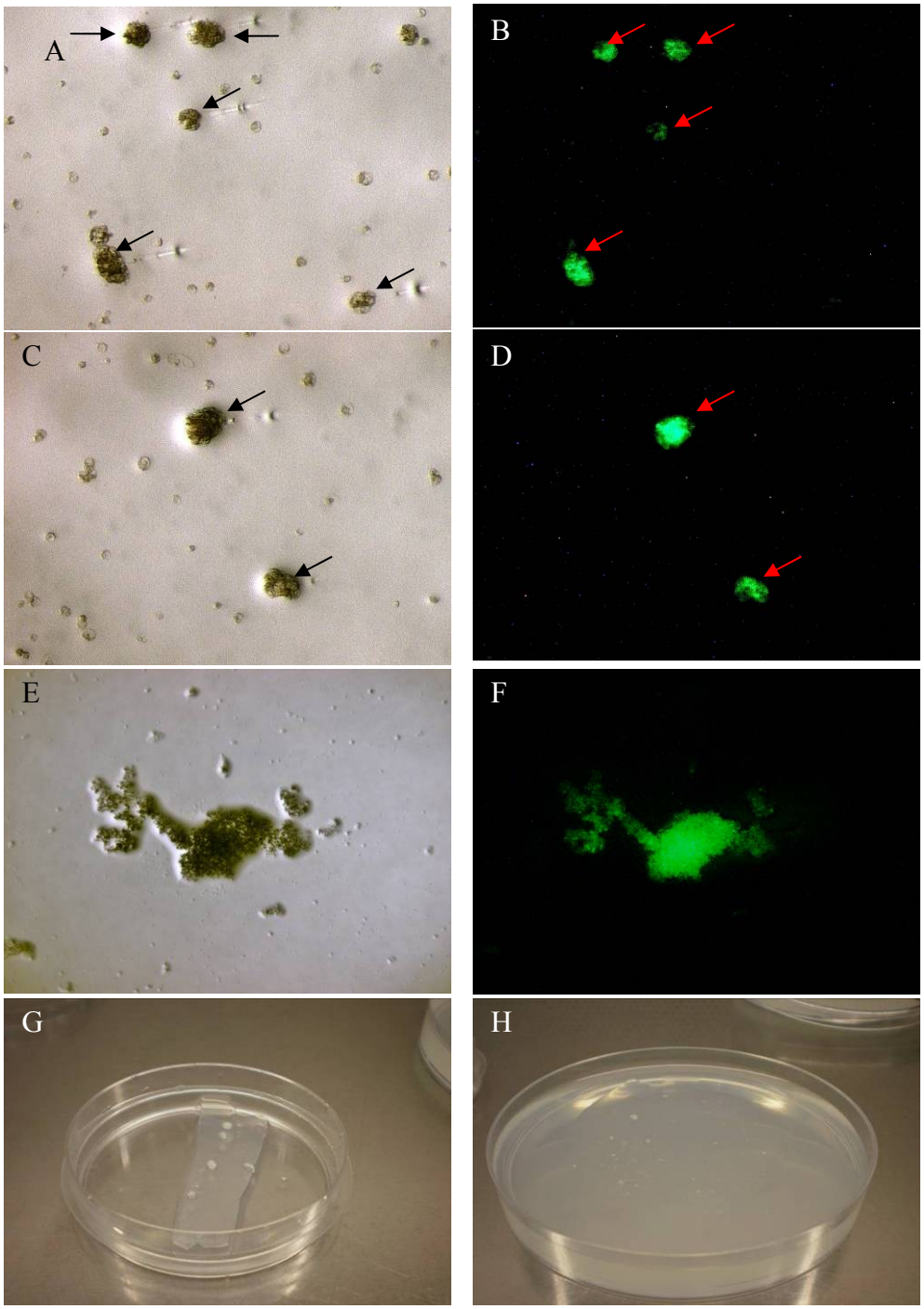

Figure III.33: Development of microcalli expressing GFP gene.

After 5 days of DNA microinjection, the alginate layer was transferred into the Y3A liquid medium consisted of 5.5 % (w/v) sucrose and 8.2 % (w/v) glucose supplemented with 10 µM NAA, 2 µM 2,4-D, 2 µM IBA, 2 µM GA3, 2 µM 2iP and 200 mg/l ascorbic acid and then were cultured at 28^{o}C for 2 weeks. Then, the medium was replaced with similar Y3A liquid medium consisted of 4 % (w/v) sucrose and 7.2 % (w/v) glucose for the development of microcolonies (A and B). The medium was then replaced with Y3A liquid medium consisted of 4 % (w/v) sucrose for the development of microcolonies (C and D) to microcalli (E and F). Then, the alginate layer containing microcalli (G) was transferred onto Y31N0.1BA solid medium (H) for plant regeneration. Arrows indicate the injected protoplasts.

IV DISCUSSION

IV.4.1 Transient expression oil palm genes in heterologous plants

The temporary or transient expression of oil palm genes such as the tissue-specific promoter and the viability of plant expression vectors in homologous or heterologous plant tissue rely only on the biolistic approach (Masura *et al.*, 2010; Masura *et al.*, 2011; Zubaidah and Siti Nor Akmar, 2010). The transient expression using biolistic transformation requires special equipment thus limited the exploration of the oil palm genes. Furthermore, preparations of different tissues from oil palm, particularly oil palm fruit is labour intensive and time consuming. For example, Zubaidah and Siti Nor Akmar (2003) developed the transient promoter assay system based on biolistic approach to determine the specificity of the oil palm MT3-B gene promoter driving GUS and GFP genes. Using this transient assay, the viability and functionality of polyhydroxybutyrate (PHB) genes construct were evaluated at the protein level in mesocarp tissue (Omidvar *et al.*, 2008). Although the results can be obtained within a week, the sampling of explant tissue such a mesocarp required almost 12 weeks and also the conditions for bombardment first need to be optimized for each type of tissues. In contrast, the PEG mediated transient expression of PIPP, D12 and HSA in tobacco protoplasts requires only three days which the results of transient expressions at the protein level. Surprisingly, similar expression efficiencies were achieved with the three different oil palm promoters LSP, MSP and KSP, demonstrating the tobacco protoplasts could be use for transient expression regulated by oil palm tissue specific promoters, particularly to evaluate the expression of recombinant protein. In addition, the application of PEG mediated transient expression in tobacco protoplasts could be extended to evaluate the expression of other recombinant proteins since most of the transient expressions were performed using infiltration through *Agrobacterium* (Kathuria *et al.*, 2002; Sriraman *et al.*, 2004).

IV.4.2 Evaluation the specificity of oil palm tissue-specific promoter in tobacco plants

The studies from transgenic tobacco plants expressing GFP gene under the control of LSP promoter demonstrated the GFP fluorescence in all green tissue, including the stem but not in the root. The expression pattern was similar to the expression of GUS gene in transgenic Arabidopsis plants (Chan and Abdullah, 2010). The expression pattern was as expected since the LSP promoter is derived from the light-harvesting chlorophyll a/b binding protein (LHCB) gene of oil palm, which was abundantly expressed in green leaf oil palm tissue, but

not in non-photosynthetic tissue such as kernel, mesocarp, germinated seedlings and inflorescences (Chan *et al.*, 2008).

MSP promoter is the promoter element for fruit-specific type 3 metallothionein-like gene (MT3-A), which was isolated from oil palm and shown to be developmentally regulated during fruit ripening (Siti Nor Akmar *et al.*, 2002). Evaluation the specificity of MSP promoter by means of GFP or GUS reporter genes revealed a strong activity in the mesocarp tissue, but low activity was also detected in the leaves of oil palm (Omidvar *et al.*, 2010). Similarly, low expression of GFP was observed in the mature leave of transgenic tobacco plants showing the MSP promoter was also regulated in heterologous plants particularly in the leaves. Meanwhile, KSP promoter is belonged to oil palm glutelin gene and the specificity of KSP promoter is clearly restricted to kernel tissue of oil palm (Abdullah *et al.*, 2008). This was verified by particle bombardment using GFP gene driven by KSP promoter which showed the GFP fluorescence spots were observed in kernel but not in the mesocarp and leaves of oil palm. However, in this study, as similar to MSP promoter, the expression of GFP was not observed in the seed or seedlings indicated the KSP and MSP promoters dominantly regulated only in the kernel and mesocarp tissues of oil palm fruits rather than in the seeds of tobacco plants. Furthermore, the suitable model plants for analysis the specificity of KSP and MSP promoters in heterologous plants is difficult to be found (Personal communication, Dr. Omar, MPOB).

IV.4.3 Potential use oil palm LSP promoter in the selection of transgenic plants

In plant genetic engineering, high expression of selectable marker usually regulated by constitutive promoters such as CaMV35S and maize ubiquitin promoter. Ideally, the constitutive promoter should regulate the selectable marker in all stage of cell development to avoid producing chimeric plants. So far, the use of a tissue-specific promoter to drive the selectable marker gene is very rare or has never been used in plant genetic engineering. In contrast to the mentioned facts, the transgenic tobacco plants producing recombinant proteins of PIPP, D12 and HSA were generated through *Agrobacterium*-mediated transformation using hygromycin selection, which hygromycin gene was control by LSP promoter. Further characterization of the transgenic tobacco plants at genomic DNA, RNA and protein levels demonstrated the stability of the transgenic plants. In addition, the transformation and plants regeneration process was comparable with the kanamycin selection which the gene expression was regulated by constitutive CaMV35 or NOS promoters. This suggests the LSP

promoter could be use in the regeneration of transgenic plants, particularly the plants produced through vegetative tissues. However, the LSP promoter cannot be use for oil palm transformation since studies from transient expression and expression profile demonstrated that LSP promoter was not active in oil palm embryogenic calli.

IV.4.4 Efficient protoplast isolation from oil palm suspension cultures

Most of the protoplast isolation from suspension cultures were based on gradient or flotation steps. The used of gradient or flotation steps in this study resulted in the low yield ($<10^2$) of protoplasts. Srisawat and Kanchanapoom (2005) failed to obtain any protoplast when they isolated the protoplasts from suspension cultures by floating in between of sucrose and sorbitol. In contrast, the present isolation protocol is very efficient for protoplast isolation from oil palm suspension cultures since it is based on optimal centrifugation steps to obtain high yield and viability and is substantially free from contamination by cell debris. In the present protocol, protoplast yield was up to 1.14×10^6 per g fwt of protoplasts. The yield was comparable to the yield of 1.5×10^6 per g fwt obtained by Sambanthamurthi *et al.* (1996), but significantly lowers than 1.0×10^7 per g fwt isolated by Te-chato *et al.* (2005). This was due to the different source of protoplasts isolated from embryogenic callus from solid media. Nevertheless, the yield of protoplast using the present protocol was significantly higher than 1×10^4 per g fwt obtained by Bass and Hughes (1984), which the protoplasts was also isolated from suspension cultures.

IV.4.5 Plant regeneration from protoplasts derived from oil palm suspension cultures

The suspension cultures have been proven to be the best source material for the successful of plant regeneration of protoplasts for many plant species. The suspension cultures are known consisting small cell clusters consisted 34-70 cells per cluster. The suspension cells are oval in shape, rich with dense cytoplasm, and consistently regenerable resulted in the efficient protoplasts isolation and further success for plant regeneration. For oil palm, Bass and Hughes (1984) was first reported protoplast isolation from suspension cultures. This was follows by Srisawat and Kanchanapoom (2005). Sambanthamurthi *et al.* (1996) and Te-chato *et al.* (2005) reported protoplast isolation of embryogenic callus from solid media, in which only microcalli were produced. In this study, the protoplasts were successfully isolated from suspension culture with cell division to form microcolonies. The microcolonies successfully grew to microcalli and, for the first time, the microcalli regenerated to plants through the process of somatic embryogenesis.

IV.4.5.1 Totipotency of protoplasts of 7 days subculture

The used of 4-8 days after subculture of suspension cultures have been proven to be the best time period for isolation of regenerable protoplast in most woody plant species. The protoplasts isolated from seven days after subculture was used for further experiment in this study due to average range of 10-15 μm of protoplast showing dense cytoplasm concentrated around the nucleus which more favourable for cell divide besides easy to handle and monitor. While 16-30 μm in sizes protoplasts were fully packed with cytoplasm showed less cell divide ability and more than 50 % of the protoplast lower than 10 μm in size having cytoplasm without nucleus. In addition, protoplasts isolated at 7 days after subculture were in the exponential phase (Teixeira *et al.*, 1995), which enabled a high degree of cell division. The high degree of cytoplasmic activity during protoplast culture and the availability of space to grow inside the 15-25 μm protoplast likely contributed to the success of plant regeneration in this study. This was shown after three days where the volume of cytoplasm increased and eventually distributed by filling the whole cell before initial the cell division. The increased volume of cytoplasm in protoplast regeneration was also observed in sugarcane (Taylor *et al.*, 1992) and wheat (Jásik *et al.*, 1992), which influenced the cell division and colony formation.

IV.4.5.2 Regenerative protoplasts of three months suspension culture

The age of suspension cultures also influenced the success in plant regeneration of oil palm protoplasts. The oil palm suspension cultures remained regenerative for at least six years (Tarmizi, 2002), theoretically during this period the protoplasts could regenerated to plant. Plant regeneration from protoplasts isolated from older than two years-old suspension cultures were achieved in wheat (Qiao *et al.*, 1992). However, the regeneration of plants from protoplasts of rice and banana is problematic, when the protoplasts were isolated from long-term maintenance of suspension cultures (Tang *et al.*, 2000; Assani *et al*., 2002). In this study, plant regeneration was achieved by using protoplasts isolated from three month old suspension cultures. Protoplasts isolated from older than three months old suspension cultures were observed having large starch granules which ruptured easily in the isolation process resulting in a decrease in protoplast yield. Furthermore, an attempt to culture using these protoplasts resulted in the no cell division and generally 80 % of the protoplasts died after 5-14 days of culture. In addition, when suspension culture was older than 3 months, it contained more compact cell clusters than friable cell clusters. Compact cell clusters were unable to undergo somatic embryogenesis. This result is in agreement with the studies of woody plant

which the friable cell clusters of suspension cultures was used for protoplast isolation and further plant regeneration.

IV.4.5.3 Y3A as optimal medium for plant regeneration of protoplasts

Efficient regeneration of plants from protoplasts for different plant species strongly depends on the media composition. The KM8P medium is frequently being used for protoplasts culture in woody plant, which was based on the medium for protoplasts culture of *Vicia hajastana* plant (Kao and Michayluk, 1975). The Y3 version media especially Y3A medium was identified as the optimum medium for protoplasts cultures in this study. This was the first time that the modified Y3 media was used for oil palm protoplasts culture. MS based media and AA media (Sambanthamurthi *et al.*, 1996) in this study was unsuccessful due to very low protoplasts division frequencies. The components of macroelements and microelements in Y3 version media were similar to original Y3 media except the potassium dihydrogen phosphate (KH_2PO_4) was added in Y3A and Y3D~Y3F media. Addition of KH_2PO_4 increased the protoplast division frequency compared to Y3 version media without KH_2PO_4 (Y3, Y3B, Y3C). In the isolation process, the protoplasts are exposed to stress and damage and when placed in the media, the adsorption of nutrients especially phosphate ions occurs intensively for the first 1-3 days to repair the damage cells (Chaillou and Chaussat, 1986). Thus, the addition of KH_2PO_4 was required to balance the nutritional requirements in all Y3 version media.

The Y3 version media contained a higher concentration of chloride ions (Cl^-) compared to the EC version media by the presence of ammonium chloride (NH_4Cl), potassium chloride (KCl) and nikel chloride ($NiCl_2.6H_20$). Although the chloride ions are known to act like natural auxins in the induction of plant root formation, they may also play an important role in the growth of oil palm protoplasts. The presence of ammonium nitrate (NH_4NO_3) in the microelements of media has been assumed to prevent the cell division of protoplasts in many woody plants such as poplar (Qiao *et al.*, 1998). In this study, protoplasts culture using media without NH_4NO_3 (ECVI medium) did not show any adverse effect but the ratio of NH_4 and NO_3 influenced the division of protoplasts. High protoplast division frequencies were obtained from Y3 version media which containing 1:4 ratio of NH_4 and NO_3 (NH_4Cl and KNO_3) compared to 1:2 ratio (NH_4NO_3 and KNO_3) in EC version media.

IV.4.5.4 Optimum PGRs for plant regeneration from protoplasts

This study determined the optimum plant growth regulators (PGRs) for successful plant regeneration from oil palm protoplasts. PGR concentrations and combinations need to be optimized for protoplast development into plants. Only two studies, citrus (Guo and Grosser, 2005) and avocado (Witjaksono *et al.*, 1998), showed the successful in plant regeneration from protoplasts culture without PGR. In this study, the continuous growth of the protoplasts was clearly affected by the combination of PGRs in each step. Efficient protoplast development to microcalli was obtained when the Y3A medium was supplemented with 3 auxins (10 µM NAA, 2 µM 2,4-D, 2 µM IBA) and 2 cytokinins (2 µM GA3, 2 µM 2iP). The low concentration of IBA, GA3 and 2iP was essential for oil palm protoplast culture as no microcalli were observed whenever these PGRs were excluded from Y3A medium. The division frequencies obtained were lower when the protoplasts were cultured in media supplemented with higher than 2 µM concentration of 2,4-D, IBA, GA3 and 2iP compared to without PGRs. Two µM each of 2,4-D, IBA, GA3 and 2iP was identified as optimum concentration for the development of microcalli from protoplasts since the frequency of cell division and formation of microcolonies and microcalli was substantially reduced when 1 µM of these PGRs was used.

IV.4.5.5 Efficient agarose bead technique for protoplasts culture

The use of suspension cultures, optimum medium and PGRs alone did not lead to the successful regeneration of plants in this study. Previous studies showed that the development of embryogenic calli from microcalli was a critical problem for oil palm protoplasts cultures. The use of agarose bead culture was identified as one of the factors in which highest frequency of formation of microcolonies (22 %) and then further development to microcalli (9.8 %) compared to protoplasts embedded in agarose solidified culture. Both culture techniques could protect and maintain the protoplast, agarose bead cultures which allowed for easy transfer and there was minimal disturbance of the protoplasts. Furthermore, the entire agarose bead was in direct contact with liquid media compared to solid media in which only the bottom part of an agarose bead was in contact with the media.

IV.4.5.6 Microcalli formation induced by reducing osmoticum

The used of liquid media with different osmotic pressures surrounding the agarose beads was identified as another factor which enhanced the development of embryogenic calli. The time points selected to change these media also influenced the growth of microcalli to

embryogenic calli. Earlier or later the time points, the more retarded the growth of protoplasts. In early protoplast culture (3 days), high osmotic pressure surrounding the agarose beads was maintained by using 21 % carbohydrate solution which was then slightly reduced by Y3A liquid medium consisting of 4 % (v/w) sucrose and 7.2 % (v/w) glucose at day 4, and reduced to normal osmotic pressure by Y3 liquid medium consisting of 4.5 % (w/v) sucrose when microcalli were observed at weeks 24.

IV.4.5.7 Ascorbic acid reduced the callus browning

The chemical reduction of PGRs and the accumulation of phenolic compounds in the agarose beads were identified as the reasons that led the microcalli browning which retarded the growth of microcalli to embryogenic calli. The use of Y35N5D2iP liquid medium supplemented with 200 mg/l ascorbid acid resulted in the development of embryogenic calli from microcalli. In contrast, the used Y35N5D2iP supplemented with $AgNO_3$ or AC did not solve the problem of browning and surprisingly the microcalli browning became worst. This could be due to $AgNO_3$ being more effective in adsorbing ethylene than the phenolic compounds. In contrast, AC adsorbed not only the phenolic compounds but also PGRs or vitamins from the media (Davey *et al.*, 2005).

IV.4.5.8 Low concentration of NAA and BA for somatic embryogenesis

Plant regeneration from protoplast-derived embryogenic calli was greatly influenced by media supplemented with low concentration of NAA and BA. Somatic embryogenesis was only observed when the agarose beads were cultured on Y31N0.1BA solid medium. Y35N5D2iP liquid medium is preferably changed to Y31N0.1BA solid medium as soon as embryogenic calli observed. Longer cultivation in Y35N5D2iP liquid medium retained the growth of embryogenic callus in the callus stage which delays the plant regeneration process. Furthermore, more CE callus was developed compared to FE callus which showed more callus multiplication than callus proliferation. Plant regeneration from protoplast-derived somatic embryos showed a similar growth pattern of plant regeneration from embryogenic calli cultures. Most of the embryos developed into normal small plantlets after subculture onto ECI1N0.1BA solid medium.

IV.4.6 Transient expression in oil palm protoplasts

Following the successful in plant regeneration from protoplasts derived from oil palm suspension cultures, it is now possible to utilize the protoplasts as starting material for the development of stable transgenic oil palms by genetic engineering. The transgene uptake subsequently integrated into the genome of oil palm protoplasts e.g. by polyethylene glycol (PEG), electroporation or microinjection could lead to the production of stable transgenic lines since those plants were regenerated from a single transformed cell. To achieve this, it is important to evaluate the suitability and viability of oil palm protoplasts to uptake DNA and subsequently give an early guide how the gene will be expressed. Since the PEG-mediated transformation is a standard method of introducing DNA into protoplasts and the result can be obtained in shorter time, the viability of oil palm protoplasts by GFP transient expression was first evaluated using this approach.

IV.4.6.1 Efficient protoplasts from suspension culture for transient GFP expression

The protoplasts isolated from oil palm suspension culture, in particular protoplasts isolated from 7 days after subculture, were identified as the most suitable for PEG-mediated transformation. The protoplasts were highly uniform in size and transfected protoplasts more easily identified. Despite no study was performed using the protoplasts isolated from green tissues such a leaf, it is expected the autofluorescences will become the major problem when dealing with GFP expression. The protoplasts isolated from suspension culture showed no autofluorescence, which would give false GFP positive. Nevertheless, the autofluorescences were still detected in protoplasts from 14 day subculture of 3 month old suspension culture and 4 month old suspension culture. The protoplasts isolated from the suspension culture should not have chloroplasts or chlorophyll, thus the autofluorescence could be due to the presence of the small amount of lipids inside protoplasts from both sources. Studies from Sambanthamurthi *et al.* (1996) showed that osmotic stress during protoplasts isolation probably induced the alteration of lipid metabolism resulting in the synthesizing of up to about 27 % palmitoleic acid.

IV.4.6.2 Optimum magnesium chloride for efficient PEG-mediated transient gene expression

The magnesium ions was identified as the important factor for the efficient PEG-mediated transient gene expression in tobacco and maize protoplasts (Negrutiu *et al.*, 1987; Maas and

Werr, 1989). In this study, ion exchanged during PEG-mediated transformation of the protoplasts greatly influenced the transfection efficiency and intensity of green fluorescence following hrGFP gene expression. The highest transfection efficiency of 2.43 % was achieved when 50 mM $MgCl_2$ was added to PEG solution compared to without $MgCl_2$. Furthermore, low to high intensity of GFP fluorescence following the concentration of 10-100 mM $MgCl_2$ indicated the increasing GFP protein accumulation. In addition, the order of ion exchanged strictly affected the PEG-mediated transformation of oil palm protoplasts. The oil palm protoplasts was exposed to Ca^{2+} ions by incubation in washing solution comprising of $CaCl_2.2H_2O$ followed by exposure to Mg^{2+} ions using PEG-MgCl solution and then again, to Ca^{2+} ions when the protoplasts-PEG solution was diluted with washing solution. The order of Ca-Mg-Ca ion was a mandatory as no GFP fluorescence was detected when the order was changed to Mg-Ca-Mg. The reason for this was unclear but most probably the negative charge of protoplasts membrane is more effective for uptake DNA when PEG interacted with Mg^{2+} ions rather than Ca^{2+} ions.

IV.4.6.3 Negative influences of DNA incubation and carrier DNA to the transfection efficiency

Longer DNA incubation time was performed in this study with the hope that the protoplasts membrane has more time in contacting plasmid DNA subsequently increase the transfection efficiency. Surprisingly, the transfection efficiency was decreased in 3 folds, 2.43 % to 0.75 % when the protoplasts were exposed to DNA for longer than 10 minutes. The PEG-MgCl solution is preferably added after 10 minutes or less of DNA incubation which is likely reduced excretion or activity of DNases from the protoplasts or cellular nucleases. Furthermore, the addition of carrier DNA which could minimize the activity of DNases or nucleases was also reduced the transfection efficiency. This suggests the inhibition of the ability of the plasmid DNA to permeabilize the protoplast membrane. The use of a high amount of DNA likely solved the problem with DNase or nucleus activities in this study. This was proven by the increasing of the transfection efficiency following the increasing of the DNA concentration.

IV.4.6.4 Toxicity of PEG and heat shock treatment as a major factors influenced the transfection efficiency

The major problem of PEG-mediated transformation of oil palm protoplasts was the toxicity of PEG. The toxicity of PEG caused the viability of the oil palm protoplasts to reduce to 50

% when higher than 25 % (v/w) PEG concentration was used. In addition, high concentration of PEG resulted in the formation protoplasts clumps or agglutinations, which leads the difficulty to identify the transfected protoplasts. Meanwhile, heat shock treatment was identified as the main reason which improved the transfection efficiency of oil palm protoplasts. It is unclear why heat shock treatment influenced the PEG-mediated transformation of oil palm protoplasts, and it could be that the protoplasts membrane was altered when incubated at 45ºC allowing for greater DNA uptake.

IV.4.7 DNA microinjection of oil palm protoplasts

Initially, an attempt to inject protoplasts embedded in agarose bead was successful but only 5 to 10 cells can be injected within an hour. This was due to the curve surface of the agarose beads resulting in difficult penetration of a needle tip at an angel of 35^{o}. The target protoplasts were not easy to identify due to the position of the protoplasts at different layer. Furthermore, the needle tips were frequently clogged or broken after only 2-3 injections probably due to agarose particles accidentally blocked the needles tip. In contrast, the alginate layer was identified as a good platform for DNA microinjection. The transparent color of alginate makes it ideal for identification of the target protoplasts and microinjection can be performed on the next target protoplast in a shorter time due to the flat surface of the alginate layer. In addition, the viability of protoplasts was maintained since no heat was applied during the preparation of alginate layer compared to agarose bead, which the agarose was maintained at 50^{o}C before mixed with protoplasts suspension. The alginate layer was also easily dissolved in sodium acetate solution to remove the developed microcalli and transferred onto the appropriate media.

The nucleus of tobacco protoplasts was identified as the efficient target compartment for DNA microinjection which the transformation frequency was at 14 % compared to 6 % with cytoplasmic microinjection (Crossway *et al.*, 1986). Further improvement of the transformation frequency at 20 % with nucleus microinjections was achieved by Schnorf *et al.* (1991). However, in this study, the nucleus was unsuitable for DNA microinjection due to the following reasons; (1) Only at least 25 μm in size of oil palm protoplasts were able for injection which will slow the injection process; (2) The technique of injection was difficult compared to cytoplasmic microinjection since the needle tip to be inserted into the protoplast must place as close as possible to the position of the nucleus; (3) Since the nucleus was surrounding by cytoplasm, the penetration of a needle tip always damaged the cytoplasm

before the needle tip could reach the nucleus; and (4) Although the needle tip reached the nucleus and subsequently performed the injection, the nucleus was swelling and moving from it natural position, in which the protoplast was often died.

The cytoplasm of protoplast injected with 100 ng/µl of DNA fragment concentration, also the use of alginate layer and lucifer yellow dye, were identified as the optimal condition for DNA microinjection of oil palm protoplasts. Since the injection was performed manually by using a microinjector CellTram vario, the amount of DNA was difficult to control thus could lead the variation of transformation frequency obtained. The dead protoplasts during microinjection were also frequently observed due to over volume of DNA solution. Nevertheless, the GFP fluorescence frequency of 78 % for 4-6 cells and 34 % for microcolonies stage indicates the decreasing transformation frequency. The decreased transformation frequency was not due to the expression of GFP but most probably the growth of protoplasts. Roughly, only 9.8 % microcalli were developed from oil palm protoplasts using agarose bead culture. In agreement with this, only 10 % transformation frequency was obtained when the injected protoplasts developed to microcalli. Although, this was the first time the DNA microinjection was used to transform oil palm protoplasts, the result indicates the transformation frequency obtained was significantly higher than 4.22 % of PEG-mediated transient expression, 1 % of biolistic (Parveez, 1998) and 0.7 % of *Agrobacterium*-mediated transformation (Izawati *et al.*, 2009) in oil palm genetic engineering.

V CONCLUSION

This study is a part during the course of MPOB and IME Board approved collaborative project "Establishment of Transgenic Oil Palm with High Added Value for commercial exploitation" (T0003050000-RB01-J). The long-term objective of this project is to generate transgenic oil palm producing recombinant proteins, PIPP (a chimeric antibody against human chorionic gonadotropin; hCG), D12 (a human antibody against dental carries) and HSA (human serum albumin), via DNA microinjection. The products will be synthesized in the leaf, mesocarp and kernel tissues of oil palm with the respects of plants must be stable and free from selectable marker.

To achieve this, the constructs of PIPP, D12 and HSA genes, which were driven either by the promoter of LSP, MSP or KSP were successful constructed and their functionality was demonstrated in tobacco plants. This will speed up the process to achieve the main objective of this project since the verification of the constructs in oil palm will take 5-10 years, particularly for the specificity in mesocarp and kernel. Upon the functionality studies, it was found that the tobacco protoplasts transient expression system could be use for exploitation of oil palm genes. The potential use oil palm LSP promoter for selection of transgenic plants through vegetative tissues was also found.

To implement the oil palm protoplast as starting material for the development of stable transgenic oil palms via DNA microinjection, the regeneration of true plants from protoplasts is a mandatory. For so long, tissue culturists were trying to regenerate oil palm from protoplasts but failed. Success in other monocot crops, such as rice, was obtained when protoplasts were isolated from suspension cultures. Therefore, an improved protocol for the efficient isolation of high-quality protoplasts from oil palm suspension cultures was established. Subsequently, by using this isolation protocol, for the first time true oil palms were successfully regenerated from oil palm protoplasts. This was achieved by optimizing a number of factors as follows:

- totipotency of protoplasts isolated from 7 days subculture
- Y3A as optimum medium for plant regeneration from protoplasts
- Optimum PGRs for plant regeneration from protoplasts
- Efficient agarose bead technique for plant regeneration from protoplasts

- Microcalli formation induced by reducing osmoticum
- Ascorbic acid reduced the callus browning
- Low concentration of NAA and BA for somatic embryogenesis

Nearly 14-17 months after protoplasts were isolated; true plants were generated using agarose bead culture. The protoplasts initiated cell wall formation in 5-7 days, first and cell division in 9-12 days, developed to microcolonies in 12-16 weeks, to microcalli in 20-24 weeks, to embryogenic calli in 28-36 weeks, to whitish and greenish embryoids in 44-52 weeks and to small plantlets in 56-68 weeks following the factors described. Further improvement such as protoplasts culture using alginate layer technique, heat shock treatments prior to protoplasts culture and the addition of ascorbic acid in the media throughout the experiment should be incorporated to accelerate the regeneration process. When the protocol is firmly developed, in the future, somatic hybridization via protoplast fusion could be carried out using this protocol to produce new traits into the oil palm.

Following the success in regeneration of plants from protoplasts, the objective of this project became clearly to be achieved in the future when the protoplasts were used for PEG-mediated transient gene expression, and further used in the stable gene expression via DNA microinjection. The efficient and reliable protocol for PEG-mediated transformation of oil palm protoplasts was developed by validating optimum parameters such as heat shock treatment, the amount of DNA, PEG and magnesium chloride concentrations, and the procedure to transfect the protoplasts. As the main objective of this study, the transgenic microcalli of oil palm were successful generated from protoplasts transformed by DNA microinjection. Since the plant regeneration from oil palm protoplasts will take over a year and four months, more conclusive results will be obtained when small plantlets are produced and analyzed. This is important before the DNA microinjection could be used to transform the recombinant gene constructs and other useful genes.

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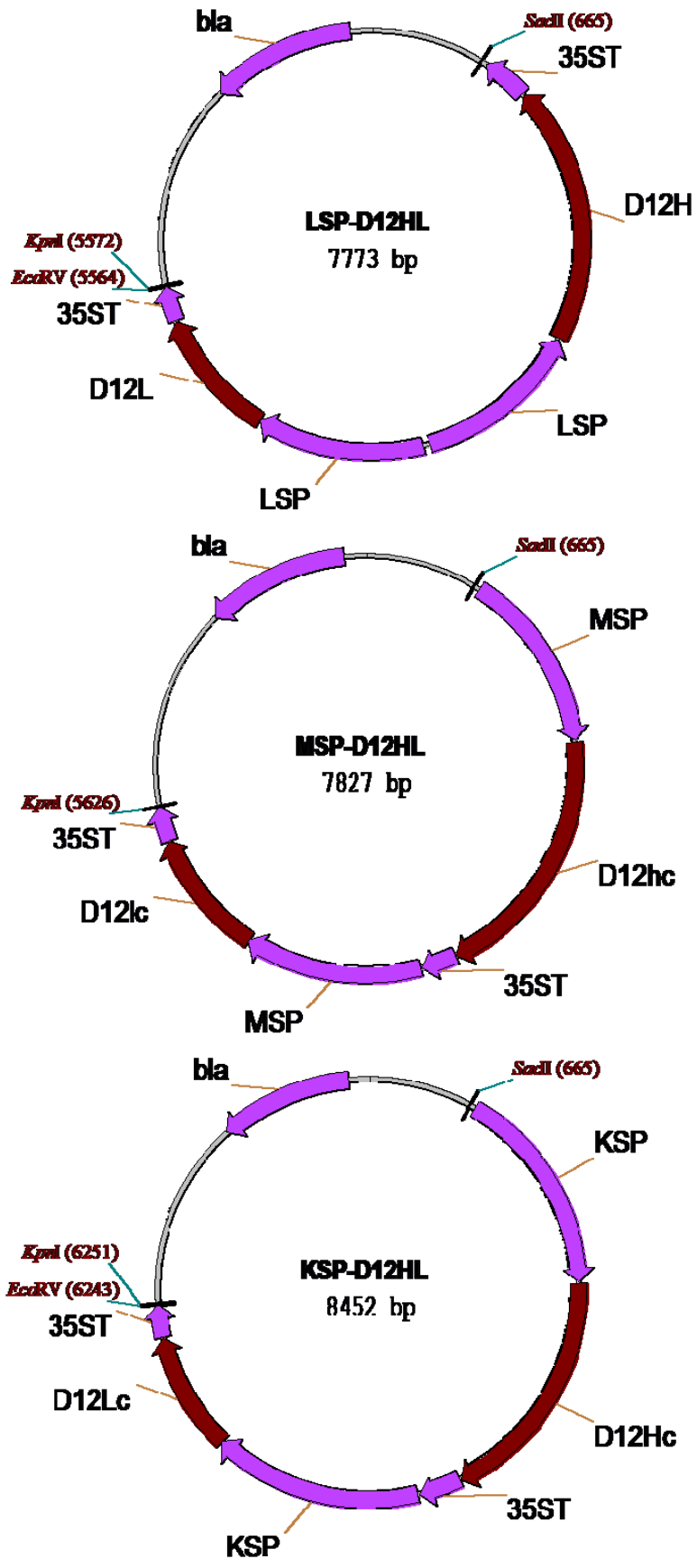
Appendix 1

Composition of the media used

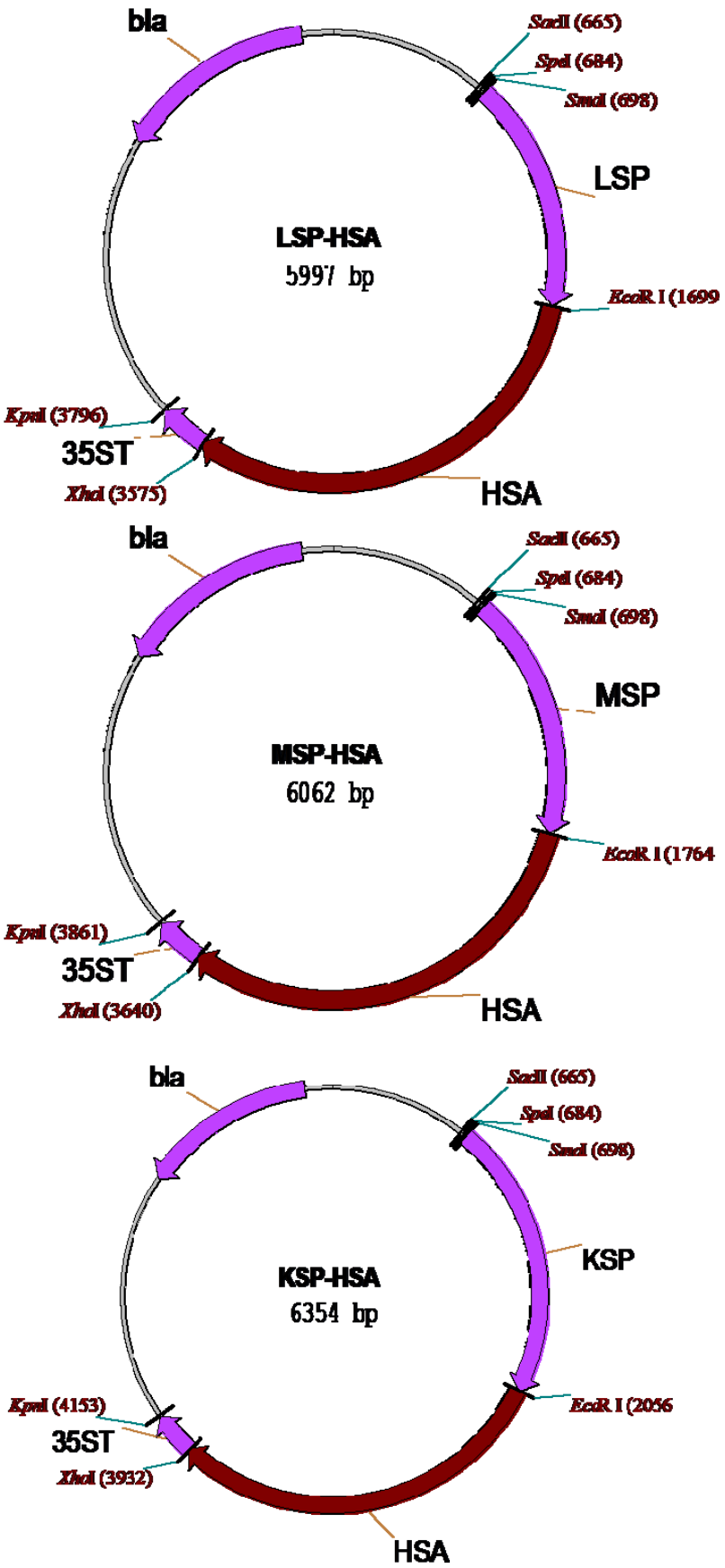
Media component	1/2ECI	ECI	ECII	ECIII	ECIV	ECV	ECVI	Y3	Y3A	Y3B	Y3C	Y3D	Y3E	Y3F
Macroelement (mg/l)														
NH_4NO_3	825	1650	600	1650	1650	1650								
NH_4Cl								535	535	535	535	535	535	535
KNO_3	950	1900	1900	1900	1900	1900	1900	2020	2020	2020	2020	2020	2020	2020
KCl			300					1492	1492	1492	1492	1492	1492	1492
$CaCl_2.2H_2O$	220	440	453	440	440	440	440	294	294	294	294	294	294	294
$MgSO_4.7H_2O$	185	370	146	370	370	370	370	247	247	247	247	247	247	247
KH_2PO_4	85	170	170	420	170	170	170		250			250	250	250
$NaH_2PO_4.H_20$								312	312	312	312	312	312	312
Microelements (mg/l)														
$MnSO_4.4H_2O$	11.15	22.3	10	22.3	22.3	22.3	22.3	11.2	11.2	11.2	11.2	11.2	11.2	11.2
$ZnSO_4.7H_2O$	4.3	8.6	2	8.6	8.6	8.6	8.6	7.2	7.2	7.2	7.2	7.2	7.2	7.2
H_3BO_3	3.1	6.2	3	6.2	6.2	6.2	6.2	3.1	3.1	3.1	3.1	3.1	3.1	3.1
Kl	0.42	0.83	0.75	0.83	0.83	0.83	0.83	8.3	8.3	8.3	8.3	8.3	8.3	8.3
$CuSO_4.5H_2O$	0.013	0.026	0.026	0.026	0.026	0.026	0.026	0.16	0.16	0.16	0.16	0.16	0.16	0.16
$CoCl_2.6H_2O$	0.013	0.026	0.026	0.026	0.026	0.026	0.026	0.24	0.24	0.24	0.24	0.24	0.24	0.24
$Na_2MoO_4.2H_2O$	0.125	0.25	0.25	0.25	0.25	0.25	0.25	0.24	0.24	0.24	0.24	0.24	0.24	0.24
$NiCl_2.6H_20$								0.0024	0.0024	0.0024	0.0024	0.0024	0.0024	0.0024
NaFeEDTA	18.75	37.5	36.7	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
Carbohydrates (g/l)														
Sucrose	30	30	30	40		5	30	45	40	40	40	30	40	29
Glucose				72		5			72			15	72	25.2
Mannitol						5								
Sorbitol						5								
Fructose						5								

Mannose						5								
Maltose						5								25
Dextrose					30	5								
Myo-inositol	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1		0.2
Vitamins (mg/L)														
Thiamine HCL	1	1	1	1	1	1	1	1	1	2.5	1	1	1	1
Pyridoxine HCL	1	1	1	1	1	1	1	1	1	0.4	1	1	1	1
Nicotinic HCL	1	1	1	1	1	1	1	1	1	10	1	1	1	1
Nicotinamide													1	1
Ca-Pantothenate													1	1
Biotine													0.1	0.1
p-Aminobenzoic														0.5
Choline chloride														0.5
Ascorbic acid										250				
Amino acids (mg/L)														
L-Glutamine	50	100	100	100	100	100	100	100	100	100	200	200	100	100
L-Asparagine	50	100	100	100	100	100	100	100	100		100			
L-Alginine	50	100	100	100	100	100	100	100	100		100			
BSA				260										
Glycine							4	4			4			
PVP-40										5000				
L-Cysteine										500				
Other organics (mg/L)														
MES	250	250	250	250	250	250	250	250	250	250	250	250	250	250
PEG4000													250	

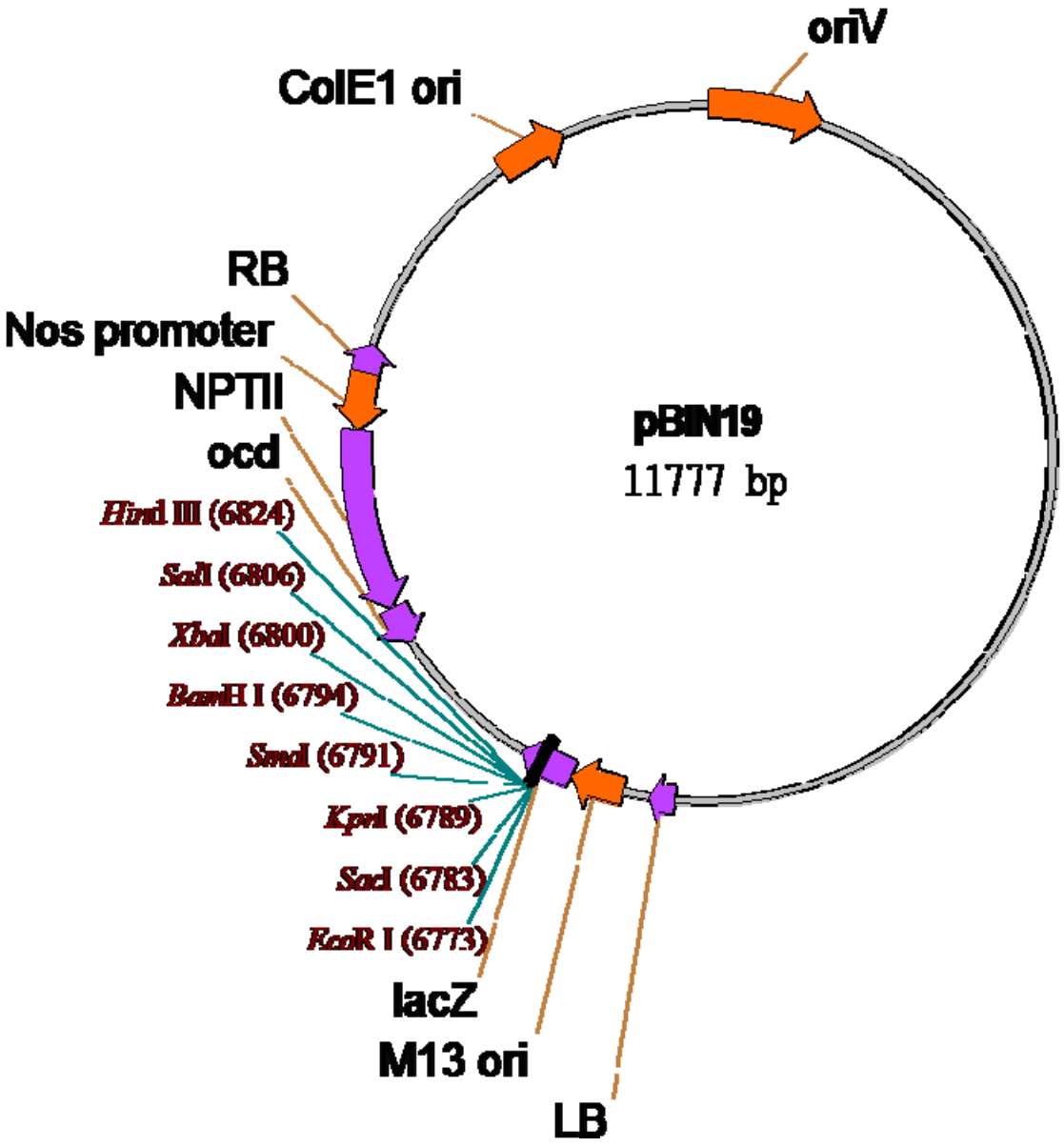
Appendix 2


bla
SacII (665)
35ST
D12H
LSP-D12HL
7773 bp
KpnI (5572)
EcoRV (5564)
35ST
D12L
LSP
LSP
bla
SacII (665)
MSP
MSP-D12HL
7827 bp
KpnI (5626)
35ST
D12hc
D12lc
35ST
MSP
bla
SacII (665)
KSP
KSP-D12HL
8452 bp
KpnI (6251)
EcoRV (6243)
35ST
D12Hc
D12Lc
35ST
KSP

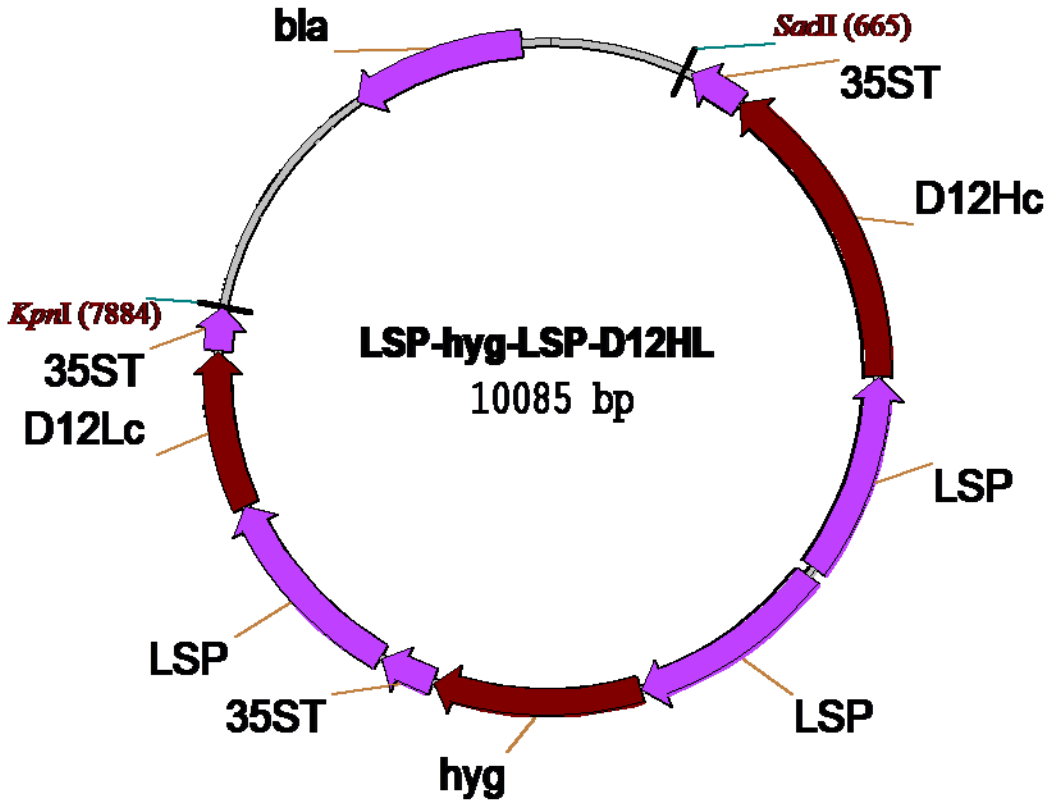
Appendix 3

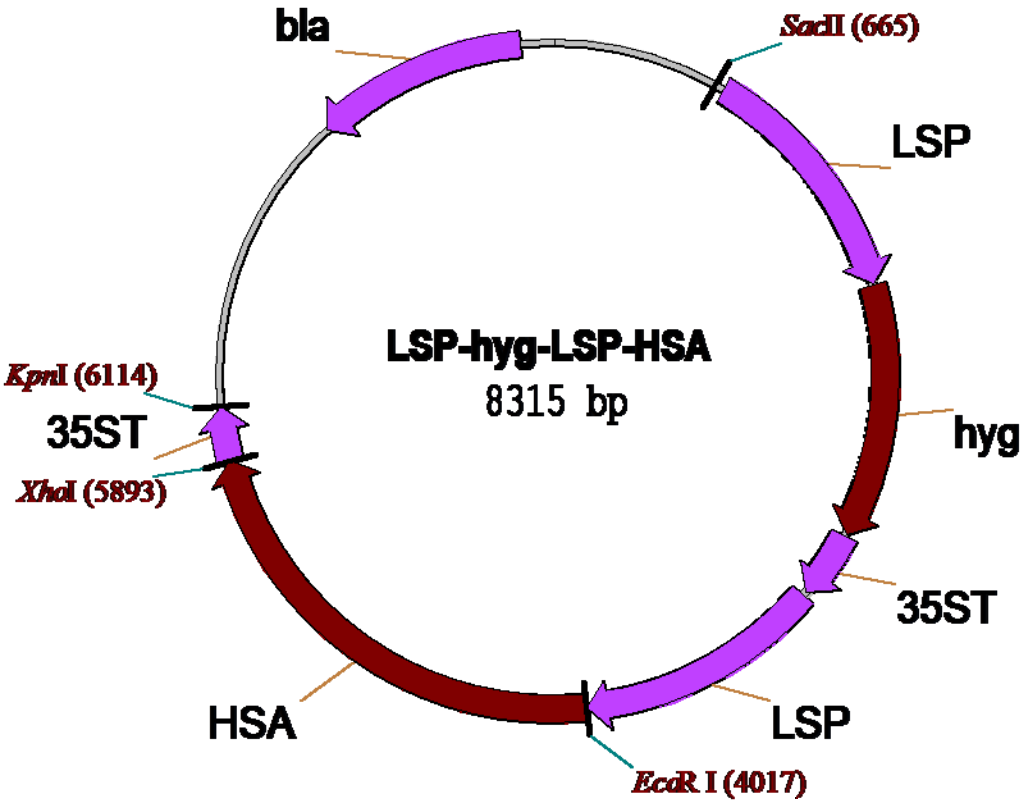

bla
SacII (665)
SpeI (684)
SmaI (698)
LSP
LSP-HSA
5997 bp
EcoR I (1699
KpnI (3796)
35ST
XhoI (3575)
HSA
bla
SacII (665)
SpeI (684)
SmaI (698)
MSP
MSP-HSA
6062 bp
EcoR I (1764
KpnI (3861)
35ST
XhoI (3640)
HSA
bla
SacII (665)
SpeI (684)
SmaI (698)
KSP
KSP-HSA
6354 bp
EcoR I (2056
KpnI (4153)
35ST
XhoI (3932)
HSA

Appendix 4


oriV
ColE1 ori
RB
Nos promoter
NPTII
ocd
pBIN19
11777 bp
HindIII (6824)
SalI (6806)
XbaI (6800)
BamH I (6794)
SmaI (6791)
KpnI (6789)
SacI (6783)
EcoR I (6773)
lacZ
M13 ori
LB

Appendix 5


bla
SacII (665)
35ST
D12Hc
LSP
LSP
35ST
hyg
LSP
KpnI (7884)
35ST
D12Lc
LSP-hyg-LSP-D12HL
10085 bp


bla
SacII (665)
LSP
hyg
35ST
LSP
EcoR I (4017)
HSA
KpnI (6114)
35ST
XhoI (5893)
LSP-hyg-LSP-HSA
8315 bp

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