

UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF CANDIDATE MARKERS FOR CALLOGENESIS IN OIL PALM (*ELAEIS GUINEENSIS* JACQ.)

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By

CONIE TOH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

November 2014

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Dedication to my beloved parents, family and friends

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

MOLECULAR CHARACTERIZATION OF CANDIDATE MARKERS FOR CALLOGENESIS IN OIL PALM (*ELAEIS GUINEENSIS* JACQ.)

By

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November 2014

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Oil palm (*Elaeis guineensis* Jacq.) is a monocotyledonous plant of the palm family (Arecaceae). Cloning of oil palm via micropropagation technique enables the improvement of planting materials using existing individuals which have most of the desired qualities such as good oil yield and composition, small height increment, drought tolerance and disease resistance. Cloning also circumvent the long generation time required with conventional breeding techniques to generate high quality elite oil palm planting material. Usually, oil palm micropropagation is performed by inducing somatic embryogenesis on calli derived from leaf tissue. However, the callogenesis rate in oil palm is very poor. A previous study had successfully isolated and identified differentially expressed sequences during oil palm callogenesis using representational difference analysis (RDA) approach. These differentially expressed sequences are potential candidate markers for oil palm callogenesis. As such, they can be used for screening explants with high callusing rates. Early identification would enable reductions in time and costs in the micropropagation process. Prior to that, these markers had to be characterized first in order to determine their applicability in predicting tissues that have potential in callusing. As such, eight candidate markers were selected for full length cDNA isolation and molecular characterization, namely gibberellin regulated protein (EgGA), nuclear transcription factor Y subunit C (EgNTF), integral membrane family protein (EgIMP), hypothetical protein (EgHypothetical), glutathione S-transferase (EgGST), glycine-rich RNA-binding protein (EgGRP), ATP-dependent Clp protease (EgClpP) and early nodulin 93 protein (EgENOD). Real time RT-PCR indicated that overall, the transcripts were found to be preferentially expressed in tissue culture derived materials from leaf (leaf explant, embryogenic callus, non-embryogenic callus, cell suspension culture, globular, haustorium and germinating embryoid) and relatively low levels in non-tissue culture derived materials (female flower, male flower, meristem and root). The expression analysis via real time RT-PCR using various leaf explants samples also indicated that four out of eight markers (EgGA, EgHypothetical, EgGST and EgClpP) had different expression pattern between the low and highly callusing tissues at certain stages, which indirectly showed that it has the capability in differentiating both tissues and thus, were characterized further via RNA *in situ* hybridization. Interestingly, all of the four markers appeared to have display a tissue-specific expression pattern. In conclusion, the expression patterns of all of the eight callogenesis-related

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transcripts are considered to be stage-dependent and genotype-specific, and are postulated to play significant roles at different stages of oil palm callogenesis. A regression model for callogenesis with a predictive accuracy of 21.6% was constructed for the EgClpP expression marker. It may be interesting to further explore EgClpP expression profiles across a wider range of oil palm genotypes in order to confirm the suitability as putative marker for screening ortets that are amenable to tissue culture.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN MOLEKUL CALON-CALON PENANDA UNTUK KALUGENESIS DALAM KELAPA SAWIT (*ELAEIS GUINEENSIS* JACQ.)

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Kelapa sawit (*Elaeis guineensis* Jacq.) adalah tumbuhan monocotyledonous keluarga sawit (Arecaceae). Pengklonan kelapa sawit melalui teknik mikropropagasi membolehkan peningkatan penanaman kelapa sawit yang menggunakan individu-individu yang mempunyai sebahagian ciri-ciri yang dikehendaki seperti hasil dan komposisi minyak yang baik, ketinggian rendah, toleransi terhadap kemarau dan daya ketahanan terhadap penyakit. Pengklonan juga dapat mengelakkan masa generasi lama yang diperlukan jika dibandingkan dengan teknik-teknik penanaman konvensional dalam menghasilkan kualiti tertinggi tanaman kelapa sawit. Biasanya, mikropropagasi kelapa sawit dilakukan dengan induksi embriogenesis somatik pada kalus yang berasal dari tisu daun. Akan tetapi, kadar kalugenesis di kelapa sawit adalah rendah. Kajian terdahulu telah berjaya mengasing dan mengenalpasti gen-gen yang diekspres secara berbeza pada peringkat-peringkat yang berlainan semasa kalugenesis melalui analisis perwakilan berbeza (RDA). Gen-gen yang diekspres secara berbeza ini adalah berpotensi untuk menjadi calon penanda kalugenesis. Oleh itu, gen-gen tersebut boleh digunakan untuk penyaringan eksplan dengan kadar kalugenesis yang tinggi. Pengenalpastian awal akan membolehkan pengurangan masa dan kos dalam proses mikropropagasi. Sebelum itu, penanda-penanda tersebut hendaklah dicirikan terlebih dahulu untuk menentukan penggunaan mereka untuk meramalkan tisu-tisu yang mempunyai potensi dalam kalugenesis. Oleh itu, lapan calon-calon penanda telah dipilih untuk pengasingan jujukan lengkap cDNA dan pencirian molekul, iaitu EgGA, EgNTF, EgIMP, EgHypothetical, EgGST, EgGRP, EgClpP dan EgENOD. *Real-time RT-PCR* menunjukkan bahawa secara keseluruhannya, transkrip menunjukkan peningkatan ekspresi dalam tisu-tisu daripada kultur tisu hasilan daun (eksplan daun, kalus embriogenik, kalus tidak embriogenik, kultur suspensi sel, globular, haustorium dan cambahan embriod) dan pengurangan ekspresi dalam tisu-tisu bukan daripada kultur tisu hasilan daun (bunga betina, bunga jantan, meristem dan akar). Analisis ekspresi melalui *real-time RT-PCR* menggunakan pelbagai sampel eksplan daun juga menunjukkan bahawa empat daripada lapan penanda-penanda (EgGA, EgHypothetical, EgGST dan EgClpP) mempunyai pola ekspresi berbeza di antara tisu-tisu yang berkadar kalugenesis rendah dan tinggi pada peringkat tertentu, yang secara tidak langsung menunjukkan bahawa penanda-penanda tersebut mempunyai keupayaan dalam membezakan kedua-dua tisu, dan oleh itu, dicirikan lagi melalui kajian hybridisasi secara *in situ* RNA. Menariknya, kesemua

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empat penanda-penanda kelihatan mempunyai paparan corak ekspresi tisu-specifik. Kesimpulannya, corak ekspresi kesemua lapan transkrip-transkrip berkaitan kalugenesis bergantung kepada peringkat dan genotaip tertentu, dan dipostulasikan dalam memainkan peranan penting pada peringkat yang berbeza dalam kalugenesis kelapa sawit. Model regresi untuk kalugenesis dengan ketepatan ramalan sebanyak 21.6% dibina penanda EgClpP. Ia mungkin menarik untuk menerokai profil ekspresi EgClpP merentasi pelbagai jenis genotaip kelapa sawit berlainan bagi mengesahkan kesesuaian EgClpP sebagai penanda putatif penyaringan ortet-ortet untuk kultur tisu.

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xiii
LIST OF FIGURES		xv
LIST OF ABBREVIATIONS		xix
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	3
2.1	*Elaeis guineensis* Jacq.	3
2.1.1	Background	3
2.1.2	Economic importance of *Elaeis guineensis* Jacq.	5
2.2	Oil palm tissue culture	8
2.2.1	Progress in oil palm tissue culture	10
2.2.2	The importance of oil palm tissue culture	11
2.2.3	Bottlenecks in oil palm tissue culture	13
2.3	Molecular markers	13
2.3.1	Somatic embryogenesis-related genes	16
2.3.2	Previously isolated callogenesis-related transcripts	17
3	**MATERIALS AND METHODS**	18
3.1	Plant Materials	18
3.2	Isolation of Full-length Sequences of Callogenesis-related cDNAs from *Elaeis guineensis* Jacq.	20
3.2.1	Synthesizing 5'- and 3'-RACE-ready cDNAs	20
3.2.2	Primer Design	26
3.2.3	Full-length Isolation of Callogenesis-related Transcripts	26
3.2.4	Gel Purification of PCR product	30
3.2.5	Cloning and Sequence Analysis	31
3.2.5.1	Cloning and Transformation	31
3.2.5.2	Colony PCR	32
3.2.5.3	Purification of plasmids	32
3.2.5.4	Sequence analysis	33
3.3	Expression Study by using real-time RT-PCR (Reverse Transcriptase-PCR)	33

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3.3.1 Expression of callogenesis-related transcripts in leaf explants with different callusing ability 33
3.3.2 Extraction of total RNA 34
3.3.2.1 RNA Quantification 35
3.3.2.2 Formaldehyde Agarose Gel Electrophoresis 35
3.3.3 Synthesis of first strand cDNA 36
3.3.4 Endogenous controls selection 36
3.3.5 Primers for real-time RT-PCR 37
3.3.6 Optimization of real-time RT-PCR run conditions 37
3.3.7 Relative Quantification (Comparative C_T Method) 40
3.3.8 Statistical analyses 41
3.4 RNA *in situ* hybridization 41
3.4.1 Single-stranded complementary RNA probes (riboprobes) preparation 41
3.4.1.1 Design of Primers 41
3.4.1.2 Template preparation 42
3.4.1.3 *In vitro* transcription 42
3.4.1.4 Dot-blot assay 44
3.4.2 Preparation of Poly-L-Lysine coated slides 45
3.4.3 Tissue fixation, embedding and sectioning 45
3.4.4 Pre-hybridization treatment and hybridization 46
3.4.5 Washing, detection and imaging 46
3.4.6 Histological staining 47

4 RESULTS AND DISCUSSION 48
4.1 Extraction of total RNA 48
4.2 Isolation of Full-length Callogenesis-related Transcripts 50
4.2.1 Sequence Analysis of EgGA (0 9 Transcript) 51
4.2.2 Sequence Analysis of EgNTF (4 10 Transcript) 58
4.2.3 Sequence Analysis of EgIMP (4 19 Transcript) 65
4.2.4 Sequence Analysis of EgHypothetical (6 21 Transcript) 72
4.2.5 Sequence Analysis of EgGST (6 22 Transcript) 79
4.2.6 Sequence Analysis of EgGRP (14 11 Transcript) 87
4.2.7 Sequence Analysis of EgClpP (14 22 Transcript) 94
4.2.8 Sequence Analysis of EgENOD (NC 2 Transcript) 103
4.3 Gene Expression Study of Callogenesis-related Transcripts 110
4.3.1 PCR Amplification Efficiency 110
4.3.2 Real-time RT-PCR quality control 114
4.3.3 Expression Profiles Study of Callogenesis-related Transcripts 114

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4.3.3.1 EgGA 116
4.3.3.2 EgNTF 125
4.3.3.3 EgIMP 129
4.3.3.4 EgHypothetical 132
4.3.3.5 EgGST 139
4.3.3.6 EgGRP 147
4.3.3.7 EgClpP 150
4.3.3.8 EgENOD 155
4.3.4 Statistical analyses 158

5 SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH 160

REFERENCES 163
APPENDICES 196
Appendix A: Formulations for Media and Solutions 196
Appendix B: Details of samples used in real-time RT-PCR (Reverse Transcriptase-PCR) study 198
Appendix C: Sequences of isolated full-length callogenesis-related transcripts 199
Appendix D: Dissociation curves of target transcripts 205
Appendix E: BLASTX and BLASTN results of transcripts 211
Appendix F: Amplified cDNA by gradient PCR at different annealing temperature 224
Appendix G: Summary for RNA *in situ* hybridization 227
Appendix H: Histology of oil palm tissues 229
BIODATA OF STUDENT 230
LIST OF PUBLICATION 231

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LIST OF TABLES

Table			Page
3.1	:	Primers Sequences for the Isolation of Full-length Transcripts.	21
3.2	:	Thermal Cycling Conditions to Isolate ORF, 5'- and 3'-end cDNAs.	28
3.3	:	Callusing rate.	34
3.4	:	List of primers sequences used in real-time RT-PCR, the expected sizes of amplicons, first strand cDNA samples selected for the optimization of annealing temperatures (gradient PCR) and the amplification efficiency tests as well as the optimized annealing temperature for individual primer pairs.	38
3.5	:	List of riboprobes used in RNA *in situ* hybridization, the initial length, the final length and the calculated carbonate hydrolysis time.	44
4.1	:	Absorbance and yield of total RNA extracted from leaf explants at different stages of callogenesis, FEC and NC.	49
B.1	:	Details of samples used in real-time RT-PCR study.	198
E.1	:	BLASTX results of EgGA.	211
E.2	:	BLASTN results of EgGA.	212
E.3	:	BLASTX results of EgNTF.	212
E.4	:	BLASTN results of EgNTF.	213
E.5	:	BLASTX results of EgIMP.	213
E.6	:	BLASTN results of EgIMP.	214
E.7	:	BLASTX results of EgHypothetical.	215
E.8	:	BLASTN results of EgHypothetical.	216
E.9	:	BLASTX results of EgGST.	216
E.10	:	BLASTN results of EgGST.	217
E.11	:	BLASTX results of EgGRP.	218

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E.12 : BLASTN results of EgGRP. 219

E.13 : BLASTX results of EgClpP. 220

E.14 : BLASTN results of EgClpP. 221

E.15 : BLASTX results of EgENOD. 222

E.16 : BLASTN results of EgENOD. 223

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LIST OF FIGURES

Figure			Page
2.1	:	Oil palm (*Elaeis guineensis* Jacq.) trees.	4
2.2	:	Expected trends in world population and edible use of vegetable oil, with demand estimated from population and per capita consumption.	7
3.1	:	Plant materials of *Elaeis guineensis* Jacq. that were used as samples.	19
4.1	:	Total RNA (1 μg) electrophoresed on a 1% (w/v) formaldehyde agarose gel.	50
4.2	:	The PCR Product of ORF of EgGA Amplified Using the 5'-RACE-Ready cDNA of SMARTer^{TM}-RACE PCR.	52
4.3	:	The Nucleotide and Deduced Amino Acid Sequence of EgGA.	53
4.4	:	Alignment of Deduced Amino Acid Sequences of EgGA.	56
4.5	:	Phylogenetic Relationship of EgGA and Different Plant Species Based on the Deduced Amino Acid Sequences.	57
4.6	:	The PCR Product of EgNTF Amplified Using the 5'-RACE-Ready cDNA of SMARTer^{TM}-RACE PCR.	59
4.7	:	The Nucleotide and Deduced Amino Acid Sequence of EgNTF.	60
4.8	:	Alignment of Deduced Amino Acid Sequences of EgNTF.	63
4.9	:	Phylogenetic Relationship of EgNTF and Different Plant Species Based on the Deduced Amino Acid Sequences.	64
4.10	:	The PCR Product of ORF of EgIMP Amplified Using the 5'-RACE-Ready cDNA of SMARTer^{TM}-RACE PCR.	66
4.11	:	The Nucleotide and Deduced Amino Acid Sequence of EgIMP.	67
4.12	:	Alignment of Deduced Amino Acid Sequences of EgIMP.	70
4.13	:	Phylogenetic Relationship of EgIMP and Different Plant Species Based on the Deduced Amino Acid Sequences.	71
4.14	:	The PCR Product of ORF of EgHypothetical Amplified Using the	73

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5'-RACE-Ready cDNA of SMARTer™-RACE PCR.

4.15 : The Nucleotide and Deduced Amino Acid Sequence of EgHypothetical. 74

4.16 : Alignment of Deduced Amino Acid Sequences of EgHypothetical. 77

4.17 : Phylogenetic Relationship of EgHypothetical and Different Plant Species Based on the Deduced Amino Acid Sequences. 78

4.18 : The PCR Product of ORF of EgGST Amplified Using the 5'-RACE-Ready cDNA of SMARTer™-RACE PCR. 80

4.19 : The Nucleotide and Deduced Amino Acid Sequence of EgGST. 81

4.20 : Alignment of Deduced Amino Acid Sequences of EgGST. 85

4.21 : Phylogenetic Relationship of EgGST and Different Plant Species Based on the Deduced Amino Acid Sequences. 86

4.22 : The PCR Product of ORF of EgGRP Amplified Using the 5'-RACE-Ready cDNA of SMARTer™-RACE PCR. 88

4.23 : The Nucleotide and Deduced Amino Acid Sequence of EgGRP. 89

4.24 : Alignment of Deduced Amino Acid Sequences of EgGRP. 92

4.25 : Phylogenetic Relationship of EgGRP and Different Plant Species Based on the Deduced Amino Acid Sequences. 93

4.26 : The PCR Product of ORF of EgClpP Amplified Using the 5'-RACE-Ready cDNA of SMARTer™-RACE PCR. 95

4.27 : The Nucleotide and Deduced Amino Acid Sequence of EgClpP. 96

4.28 : Alignment of Deduced Amino Acid Sequences of EgClpP. 100

4.29 : Phylogenetic Relationship of EgClpP and Different Plant Species Based on the Deduced Amino Acid Sequences. 102

4.30 : The PCR Product of ORF of EgENOD Amplified Using the 5'-RACE-Ready cDNA of SMARTer™-RACE PCR. 104

4.31 : The Nucleotide and Deduced Amino Acid Sequences of EgENOD. 105

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4.32 : Alignment of Deduced Amino Acid Sequences of EgENOD. 108

4.33 : Phylogenetic Relationship of EgENOD and Different Plant Species Based on the Deduced Amino Acid Sequences. 109

4.34 : Real-time RT-PCR efficiency plots of C_T values generated versus the log input of template amounts. 111

4.35 : Expression Profiles of EgGA. 117

4.36 : Localization of mRNA transcript of elongation factor (*ELF*, positive control) (1) and EgGA (2) in globular embryo hybridized with antisense (A) and sense (B) of each riboprobe. 121

4.37 : Localization of mRNA transcript of elongation factor (*ELF*, positive control) (1) and EgGA (2) in germinating embryo hybridized with antisense (A) and sense (B) of each riboprobe. 122

4.38 : Localization of mRNA transcript of elongation factor (*ELF*, positive control) (1) and EgGA (2) in oil palm female flower hybridized with antisense (A) and sense (B) of each riboprobe. 123

4.39 : Localization of mRNA transcript of elongation factor (*ELF*, positive control) (1) and EgGA (2) in oil palm leaf explants hybridized with antisense (A) and sense (B) of each riboprobe. 124

4.40 : Expression Profiles of EgNTF. 126

4.41 : Expression Profiles of EgIMP. 130

4.42 : Expression Profiles of EgHypothetical. 133

4.43 : Localization of mRNA transcript of elongation factor (*ELF*, positive control) (1) and EgHypothetical (2) in embryogenic callus hybridized with antisense (A) and sense (B) of each riboprobe. 136

4.44 : Localization of mRNA transcript of EgHypothetical in globular embryo hybridized with antisense (A) and sense (B) of each riboprobe. 137

4.45 : Localization of mRNA transcript of EgHypothetical in germinating embryo hybridized with antisense (A) and sense (B) of each riboprobe. 137

4.46 : Localization of mRNA transcript of EgHypothetical in oil palm female flower hybridized with antisense (A) and sense (B) of each riboprobe. 138

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4.47 : Expression Profiles of EgGST. 140

4.48 : Localization of mRNA transcript of EgGST in germinating embryo hybridized with antisense (A) and sense (B) of each riboprobe. 144

4.49 : Localization of mRNA transcript of EgGST in embryogenic callus hybridized with antisense (A) and sense (B) of each riboprobe. 144

4.50 : Localization of mRNA transcript of EgGST in oil palm leaf explants hybridized with antisense (A) and sense (B) of each riboprobe. 145

4.51 : Localization of mRNA transcript of elongation factor (*ELF*, positive control) (1) and EgGST (2) in non-embryogenic callus hybridized with antisense (A) and sense (B) of each riboprobe. 146

4.52 : Expression Profiles of EgGRP. 148

4.53 : Expression Profiles of EgClpP. 151

4.54 : Localization of mRNA transcript of EgClpP in oil palm leaf explants hybridized with antisense (A) and sense (B) of each riboprobe. 154

4.55 : Localization of mRNA transcript of EgClpP in germinating embryo hybridized with antisense (A) and sense (B) of each riboprobe. 154

4.56 : Expression Profiles of EgENOD. 156

4.57 : Fitted Line Plot of relative transcript levels of EgClpP versus callogenesis rate. 159

F.1 : Amplified (A) EgHypothetical cDNA, (B) EgENOD cDNA and (C) EgNTF cDNA by gradient PCR at different annealing temperature ranging from 47°C to 57°C. 224

F.2 : Amplified EgGST cDNA by gradient PCR at different annealing temperature ranging from 49°C to 64°C. 225

F.3 : Amplified (A) EgGRP cDNA, (B) EgIMP cDNA, (C) EgGA cDNA and (D) EgClpP cDNA by gradient PCR at different annealing temperature ranging from 50°C to 65.8°C. 226

H.1 : Histology of different oil palm tissues. 229

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LIST OF ABBREVIATIONS

α	alpha
β	beta
λ	lambda
μg	microgramme
μl	microliter
°C	degree centrigrade
%	percentage
Amp	ampicillin
bp	base pair
BLAST	basic local alignment search tool
BSA	bovine Serum Albumin
cDNA	complementary DNA
cDNA-RDA	cDNA-representational difference analysis
cm	centimeter
C_T	threshold cycle
DNA	deoxyribonucleic acid
Dnase I	deoxyribonuclease 1
dNTPs	deoxynucleoside triphosphates
dATP	2'-deoxy-adenosine-5 '-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5 '-triphosphate
dTTP	thymidine-5'-tryphosphate
dH_2O	distilled water
DEPC	dietyl pyrocarbonate
DTT	dithiothreitol
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis-(β-aminoethyle ether)
EST	expressed sequence tag
EtBr	ethidium bromide
g	gramme
GSPs	gene specific primers
GTE	glucose-Tris-EDTA
hr	hour
IPTG	isopropylthio-β-D-galactoside
Jacq.	Jacquin
kb	kilobase-pair
LB	luria-bertani
LiCl	lithium chloride
M	molarity
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate

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min	minute(s)
mm	millimeter
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MPOB	Malaysian Palm Oil Board
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	nanogramme
OD	optical density
ORF	open reading frame
PCI	phenol : chloroform : isoamyl
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
PVPP	polypolyvinylpyrrolidone
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SSC	sodium chloride-sodium citrate buffer
TAE	tris acetate EDTA
TE	tris-HCL-EDTA
T_m	melting temperature
UPM	universal primer A mix
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION

Malaysia is currently the world's second largest producer and exporter of oil palm after Indonesia, with Malaysia and Indonesia together accounting for 85% of world palm oil production (Oil World Annual, 2012). World vegetable oil production has increased continuously in the past decades, with the main growth has been in palm oil, accounting for 31.3% of vegetable oil production in 2011 (Oil World Annual, 2012). The total exports of oil palm products in Malaysia, consisting of palm oil, palm kernel oil, palm kernel cake, oleochemicals, biodiesel and finished products increased by 5.3% or 1.21 million tonnes to 24.27 million tonnes in 2011 from 23.06 million tonnes recorded in 2010 (MPOB, 2011). This shows that the palm oil industry is an important component of the national economy, especially the agriculture sector.

In Malaysia, the success of the oil palm is attributed to many factors, which include favourable climatic conditions, well-established infrastructure, management skills and technology for oil palm cultivation and a land ownership structure which favours estate type of agriculture (Basri *et al.*, 2005). With good quality planting materials and agronomic practices, oil palm begins producing the oil-bearing fruit bunches as early as two and a half years after planting. While the lifespan of oil palm, as demonstrated by specimens planted in the Bogor Botanic Garden, Indonesia, is at least 120 years, the crop is generally grown for 25 to 30 years before being replanted. This is mainly because old palm becomes too tall to harvest economically (Basri *et al.*, 2005).

Oil palm micropropagation via somatic embryogenesis (SE) is worth special attention considering its numerous applications, including clonal propagation of identical or true-to-type 'photocopies' of a selected palm (ortet), by developing plantlets from the leaf tissue of *tenera* oil palms with desired characteristics. This technique has been performed by many laboratories in oil palm industry, as it improves the planting materials using existing individuals which have all or most of the desired qualities such as good oil yield and composition, small height increment, drought tolerance and disease resistance (Zamzuri, 2011; Mutert and Fairhurst, 1999). The fact that SE can be initiated from tissues other than the shoot apex is a real asset, especially for single-stemmed species in which removing the shoot tip results in the death of the donor plant. The use of clonal palms was predicted to improve oil production up to 30% (Low *et al.*, 2008). At present, there is a potential demand for more than 100 million oil palm (*Elaeis guineensis* Jacq.) tissue cultured plantlets in the world (Corley, 2009; Sharifah and Abu, 2007).

SE typically involves callogenesis as the intermediate phase. Callus is defined as an amorphous (no definite shape) mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the cultured explants. All plant tissues are potential sources of explants for

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callus induction using micropropagation technique. Calli can be obtained from tissue fragment and have the ability to differentiate into tissues, organs and even embryos, being able to regenerate whole plants. Unlike other crops, oil palm tissue culture is a very slow process. The regeneration process through oil palm tissue culture takes 2 to 4 years depending on genotype. On average at least 18 months are required to produce complete plants from callus derived from leaf explants, with callusing rate of only about 20% for young leaf and root explants while the rate of embryogenesis from proliferating callus culture is only 3 - 6% depending on genotypes (Rajainadu *et al.*, 2007; Rohani *et al.*, 2000; Wooi, 1995), making oil palm tissue culture rather inefficient. In oil palm callogenesis, some of the callus remains compact and nodular and undergoes embryogenesis (Rohani *et al.*, 2000; Kanchanapoom and Domyoas, 1999), and this process would eventually lead to somatic embryo formation and maturation, shoot regeneration, rooting and finally the recovery of new viable plantlets. However, the callus could also form into soft, granular and translucent tissues, which do not have any embryogenic potential (Rohani *et al.*, 2000). Little is known about the molecular mechanisms involved in the processes and the gene expressed during callogenesis and embryogenesis (Low *et al.*, 2008). Thus, deciphering their molecular basis can help improve the oil palm tissue culture process, with reductions in terms of time and costs, making large-scale propagation viable. By increasing the efficiency of callus production, the possibility of producing friable embryogenic callus will also be enhanced, with the reduction in the number of ortets required for culturing and an increase in production on the *in vitro* system.

Various attempts have been undertaken to improve the efficiency of oil palm tissue culture method. One of the attempts were to identify potential molecular marker for callogenesis so that it can be used to screen explants that are amenable to tissue culture. A previous study had successfully isolated and identified differentially expressed sequences during oil palm callogenesis (0 day vs 6^{th} week, 4^{th} week vs 14^{th} week), as well as in the friable embryogenic callus in comparison to nodular non-embryogenic callus using representational difference analysis (RDA) approach (Fatihah, 2010). These differentially expressed sequences are potential candidate markers for oil palm callogenesis. As such, they can be used for screening explants with high callusing rates. Early identification would enable reductions in time and costs in the tissue culture process. Prior to that, these potential markers had to be characterized first in order to determine their applicability in predicting tissues that have high callusing properties in oil palm.

Hence, the aims of this study were

1. to isolate full length cDNA for the callogenesis-related markers
2. to perform molecular characterization, and
3. to predict their potential as markers to detect high callogenesis explants.

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LIST OF PUBLICATION

Conie, T., Parameswari, N., Ho, C.L. and Sharifah, S.R.S.A. (2015). Isolation and Characterization of EgGST, a Glutathione S-transferase Protein Transcript in Oil Palm (*Elaeis guineensis* Jacq.). *Pertanika Journal of Tropical Agricultural Science* 38(2): 235-257.

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