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

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By

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

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Dedication to my beloved parents, family and friends

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

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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

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.

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

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

LIST OF TABLES

LIST OF FIGURES

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

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

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

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.