UNIVERSITI PUTRA MALAYSIA

AN EXAMINATION OF DIFFERENTIALLY-EXPRESSED GENES FROM OIL PALM EMBRYOGENIC AND NON-EMBRYOGENIC CULTURES

OOI SIEW ENG

AN EXAMINATION OF DIFFERENTIALLY-EXPRESSED GENES FROM OIL PALM EMBRYOGENIC AND NON-EMBRYOGENIC CULTURES

By

OOI SIEW ENG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

May 2003

Dedicated to my parents

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy.

AN EXAMINATION OF DIFFERENTIALLY-EXPRESSED GENES FROM OIL PALM EMBRYOGENIC AND NON-EMBRYOGENIC CULTURES

By

OOI SIEW ENG

May 2003

Chairman: Associate Professor Harikrishna Kulaveerasingam, Ph.D.

Faculty: Food Science and Biotechnology

To date the embryogenesis rate in oil palm tissue culture averages 6%. Thus, there is a need to find ways to increase this rate and also to create a selection system that is able to distinguish embryogenically competent calli from their non-embryogenic counterpart at an early stage. Using cold plaque screening of oil palm suspension cultures, about 1000 clones were isolated. About sixty-four percent of the clones have extremely low expression levels in suspension cultures and non-embryogenic calli. Another 22% were found to be up-regulated in suspension cultures compared to non-embryogenic calli. Out of the 600 sequenced clones, 46% were found to be novel or similar to proteins with unknown functions. Most of the other genes were found to be involved in cell metabolism and proliferation, which leads to an increase in the expression of genes involved in namely, protein synthesis and signal transduction pathways.

cp919.1, a TUBBY-like protein homolog or a similar member of the TULP family is up-regulated in non-embryogenic callus. cp194.2, a member of a novel serine/threonine kinase subfamily, is up-regulated in embryogenic callus. As

hypothesized in the mammalian systems, cp194.2 may play a role in the disruption of the extracellular matrix surrounding the proembryogenic masses. Transcripts of cp664.2, a truncated leucine-rich-repeat encoding protein and cp610.2, a HD-Zip II gene were up-regulated in suspension cultures. The role of cp610.2 is unknown, but it may be involved in the transcription of genes involved in early embryogenesis. 3N42.2 was isolated by RT-PCR followed by cDNA library screening. 3N42.2, a NAC superfamily member, like *CUC1/2* may be expressed in the presumptive shoot apical meristem of the embryos and then be restricted to the boundary regions of the apical meristem at later embryogenic stages. 3N42.2 expression then remains at the boundary regions of the meristem during post-embryonic development. cp194.2, cp610.2 and 3N42.2 may be used as markers for early somatic embryogenesis. Functional elucidation needs to be carried out to understand the roles that these proteins play in plant development.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

PEMERIKSAAN EKSPRESI-BEZAAN GEN-GEN DALAM KULTUR EMBRIOGENIK DAN TIDAK EMBRIOGENIK DARIPADA KELAPA SAWIT

Oleh

OOI SIEW ENG

Mei 2003

Pengerusi: **Profesor Madya Harikrishna Kulaveerasingam, Ph.D.**

Fakulti: **Sains Makanan dan Bioteknologi**

Setakat ini, kadar embriogenesis dalam tisu kultur kelapa sawit adalah pada purata enam peratus. Oleh itu, cara-cara untuk meningkatkan kadar ini dan menghasilkan satu sistem pemilihan yang dapat membezakan kalus berkebolehan embriogenik daripada kalus tidak berkebolehan embriogenik pada peringkat awal diperlukan. Kira-kira seribu klon telah diasingkan daripada penyaringan 'cold plaque' atas kultur ampaian kelapa sawit. Lebih kurang 64% daripada klon-klon tersebut didapati mempunyai tahap ekspresi rendah dalam kultur ampaian dan kalus tidak embriogenik. Terdapat 22% yang mempunyai ekspresi tinggi dalam kultur ampaian berbanding dengan kalus tidak embriogenik. Daripada 600 klon yang dijujuk, 46% didapati baru ataupun menyerupai protein yang tidak diketahui fungsinya. Kebanyakan gen-gen yang lain didapati terlibat dalam metabolisma sel and pemperidian, yang menyebabkan peningkatan dalam ekspresi sel yang terlibat dalam sintesis protein dan laluan transduksi isyarat.

Ekspresi cp919.1, homolog protein 'TUBBY-like', atau ekpresi ahli lain daripada famili gen TULP, meningkat dalam kalus tidak embriogenik. Ekspresi cp194.2, ahli

gen daripada sub-famili baru serine/threonine kinase, didapati meningkat dalam kalus embriogenik. Serupa dengan hipotesis dalam sistem mamalia, cp194.2 mungkin berperanan dalam gangguan matriks ekstrasel yang mengelilingi gumpalan 'proembryogenic'. Profil ekspresi untuk cp664.2, sebahagian protein 'leucine-rich repeat', dan cp610.2, gen HD-Zip II, didapati meningkat dalam kultur ampaian. Peranan cp610.2 tidak diketahui, tetapi mungkin terlibat dalam transkripsi gen semasa peringkat awal embriogenesis. 3N42.2 telah diasingkan melalui RT-PCR dan kemudiannya dengan penyaringan koleksi cDNA. Transkrip-transkrip 3N42.2, ahli NAC 'superfamily', seperti *CUC1/2* mungkin ditemui di 'presumptive' pucuk apeks meristem dalam embrio dan kemudiannya dihadkan kepada bahagian sempadan meristem apeks pada peringkat lewat embriogenesis. Ekspresi 3N42.2 kemudiannya kekal berada di bahagian sempadan meristem semasa perkembangan pasca-embriogenesis. cp194.2, cp610.2 dan 3N42.2 mungkin boleh digunakan sebagai penanda untuk peringkat awal embriogenesis somatik. Kajian fungsi diperlukan untuk memahami dengan lebih mendalam tentang peranan protein-protein ini di dalam perkembangan tumbuh-tumbuhan.

ACKNOWLEDGEMENTS

My deepest heartfelt gratitude first of all goes to Dr. Harikrishna, for his guidance and advice throughout my Ph.D. My appreciation to Dr. Sharifah for her guidance, friendship and always giving some time to listen and help me with the technical difficulties and also to Dr. Tan Siang Hee for being my co-supervisor all this time. I would like to thank the Ministry of Science and Technology, UPM and the Malaysian-MIT Biotechnology Partnership Programme (MMBPP) for granting me the PASCA scholarship and funding for this project respectively.

My sincerest gratitude also goes to Dr. Meilina, my informal 'co-supervisor' for her guidance, friendship, stimulating discussions and ideas. My heartfelt thanks to Ms. Girlie Wong for her kindness and help in providing the invaluable cultures used in this project. Also to En. Azman from OPRS, En. Zamzuri and Kak Zaiton of MPOB, for their kind generosity in providing some of the materials as well.

To my lab members, who have been there to lend a helping hand many many times, Zaidah, Kak Azizah, Ayu, Kak Feshah, Ruslan and Shamsul, I am especially grateful. My deepest appreciation also goes to Komala, for being a good friend, thought-provoking discussions and help too. To my UPM colleagues, Pao Theen, Mei Chooi, Yen Yen, Radziah, Wan Chin, Siti Habsah and Yang Ping, thank you for all your help and experiences you have shared with me.

Finally to my family, especially my parents, who have been very supportive and understanding throughout the ups and downs during the course of my Ph.D.

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL SHEETS		viii
DECLARATION FORM		x
LIST OF TABLES		xiv
LIST OF FIGURES		xv
LIST OF ABBREVIATIONS		xvii
CHAPTER		
1	INTRODUCTION	1
2	LITERATURE REVIEW	
	2.1 Oil Palm – An Introduction	4
	2.2 The Oil Palm Embryo	5
	2.3 The Oil Palm Tissue Culture Process	7
	2.4 Plant Embryogenesis	12
	2.4.1 Zygotic Embryogenesis	14
	2.4.2 Somatic Embryogenesis	20
	2.4.3 Polarity in Embryogenesis	26
	2.5 Shoot Apical Meristem	28
	2.5.1 Gene Expression in the Shoot Apical Meristem	33
	2.5.2 NAC-domain Family of Proteins	40
	2.6 Homeodomain Proteins	47
	2.6.1 Plant Homeoboxes	50
	2.6.2 Homeodomain-Leucine Zipper Proteins	52
	2.7 STK16	59
3	MATERIALS AND METHODS	
	3.1 Plant Materials	62
	3.2 Extraction of Total RNA and Poly A^+ RNA	63
	3.3 Construction of the Oil Palm Suspension Culture cDNA Library	67
	3.4 Screening of the Suspension Culture cDNA Library	70
	3.4.1 *In vivo* Excision of Plaques	73
	3.4.2 Cold Plaque Screening	74
	3.5 Plasmid Miniprep	75
	3.6 Sub-cloning Procedure	76
	3.7 Reverse Northern Hybridization	77
	3.8 Reverse Transcription PCR (RT-PCR)	78
	3.9 First-strand cDNA Quantification Procedure	79
	3.10 RNA Blotting and Hybridization Procedures	80
	3.10.1 RNA Blotting	80
	3.10.2 Northern Hybridization	82

3.11 Isolation of Genomic DNA 84
3.12 Southern Blotting and Hybridization Procedures 85
3.12.1 Southern Blotting 85
3.12.2 Southern Hybridization 87
3.13 *In situ* Hybridization 87
3.13.1 Probe Preparation 87
3.13.2 Fixation of Tissue Samples and Sectioning 89
3.13.3 Hybridization, Washing and Detection 90
3.14 Histological Staining 93
3.15 *Agrobacterium*-mediated Transformation in *Arabidopsis* 93
3.15.1 TransformationVectors Construction 93
3.15.2 Transformation Procedure 94
3.16 Sequence Analysis 95

4 RESULTS
4.1 Cold Plaque Screening 96
4.1.1 Screening and Reverse Northern Hybridization 96
4.1.2 Categorization of Cold Plaque Clones 98
4.2 Analysis of Selected Cold Plaque Clones 101
4.2.1 Sequence Analysis and Preliminary Characterization of cp919.1 102
4.2.2 Sequence Analysis and Preliminary Characterization of cp664.2 107
4.3 cp194.2 112
4.3.1 Sequence Analysis 112
4.3.2 Northern and Southern Analyses 117
4.4 cp610.2 123
4.4.1 Sequence Analysis 123
4.4.2 Northern and Southern Analyses 128
4.5 NAC-domain Containing Gene (3N42.2) 134
4.5.1 Sequence Analysis 139
4.5.2 Northern and Southern Analyses 139
4.6 Histology 152
4.7 Difficulties in *in situ* Hybridization and Transformation 154

5 DISCUSSION
5.1 Cold Plaque Screening 159
5.2 Preliminary Analyses of cp919.1 and cp664.2 163
5.2.1 TUBBY-like Protein (cp919.1) 163
5.2.2 Leucine-Rich Repeat Protein (cp664.2) 168
5.3 Possible Connection of cp194.2 to Embryogenesis 170
5.4 What Possible Roles Can cp610.2 Play in Embryogenesis? 177
5.5 Does 3N42.2 Play Similar Roles as *CUC1/2* Genes in SAM Formation? 182
5.6 3N42.2 and Oil Palm Cultures of Determinate Growth 184
5.7 Future Studies 185

6 CONCLUSION 188
REFERENCES 192
APPENDICES 216
Appendix A: Putative Identities of the Differentially-expressed Cold Plaque Clones 216
Appendix B: Formulation of Media and Solutions 223
VITA 225

LIST OF TABLES

Table		Page
3.1	Types of *in vitro* cultures and their respective clones/lines provided by AAR and FELDA.	62
4.1	Clones that generated signals from each hybridization analysis with SC and NEC probes as well as combined analysis from both hybridization results.	97
4.2	Cold plaque clones with their putative identities selected for Northern analysis.	101
Appendix A	Putative identities of the differentially-expressed cold plaque clones.	216

LIST OF FIGURES

Figure		Page
2.1	Oil palm seed and early growth of seedling.	6
2.2	Oil palm fruit development.	8
2.3	The oil palm tissue culture process.	9
2.4	An overview of plant embryogenesis.	15
2.5	Radial pattern formation in the Arabidopsis embryo.	15
2.6	Zones and layers within the shoot apical meristem.	30
2.7	Schematic representation of the embryonic expression patterns of genes involved in initiation and maintenance of the SAM.	34
2.8	Schematic representation of in situ hybridization analysis for *CUC2* and *STM*.	44
2.9	Leucine zipper symmetrically positions the adjacent basic regions for DNA binding.	53
4.1	Pie chart depicting the 12 categories of the cold plaque clones in terms of percentage.	100
4.2	Pie chart depicting the 12 categories in terms of percentage of the cold plaque clones with up-regulated expression in suspension cultures.	100
4.3	Nucleotide and deduced amino acid sequences of cp919.1.	102
4.4	Sequence alignment of cp919.1 with examples of other members from TUBBY-like protein (TULP) family.	104
4.5	Northern and Southern analyses for selected cold plaque clones.	106
4.6	Sequence alignment of cp664.2 with examples of other members from LRR family of proteins.	108
4.7	Nucleotide and deduced amino acid sequences of cp194.2.	113
4.8	Sequence alignment of cp194.2 with examples of other members from the novel serine/threonine kinase subfamily.	114
4.9	Northern, Southern and hydropathy analyses for cp194.2.	118

4.10	Nucleotide and deduced amino acid sequences of cp610.2.	124
4.11	Sequence alignment of cp610.2 with examples of other members from HD-Zip family of proteins.	125
4.12	Alignment of homeodomain and leucine-zipper consensus sequences from different subfamilies with cp610.2.	127
4.13	A neighbor-joining tree of 23 HD-Zip genes with 610.2.	129
4.14	Northern, Southern and hydropathy analyses of cp610.2.	130
4.15	Alignment of 3N42.2 and 3N79.2 nucleotide sequences.	136
4.16	Nucleotide and deduced amino acid sequences of 3N42.2.	140
4.17	Sequence alignment of 3N42.2 with examples of other NAC-domain containing family members.	141
4.18	A neighbor-joining tree of 18 NAC family members with 3N42.2.	142
4.19	RT-PCR, hydropathy profile and Southern analysis of 3N42.2.	143
4.20	Determinate oil palm *in vitro* shoots.	145
4.21	Northern analyses of 3N42.2.	147
4.22	Northern analyses of 3N42.2, 18S ribosomal cDNA as loading control, histone H2B and eIF-1α at different stages of floral development.	151
4.23	Histological sections of suspension cultures from different clones and developing embryo stages.	153
4.24	Construction of transformation vectors for cp194.2 and cp610.2.	156
4.25	*In situ* hybridization of embryogenic suspension cultures with 269-10-D2 and cp610.2.	158
4.26	Schematic representation of the temporal expression profiles of cp194.2, cp610.2 and 3N42.2 in somatic embryogenesis.	190

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2-BE	ethyleneglycol monobutylether
AAR	Applied Agricultural Services Sdn. Bhd.
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
Cat.	catalog number
cDNA	copy DNA
Ci	Curie
C-terminal	carboxyl terminal
D x P	*Dura x Pisifera*
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase 1	deoxyribonuclease 1
dNTPs	deoxynucleotides
DTT	dithiothreitol
dTTP	thymidine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis- (β-aminoethyl ether)

EtBr	ethidium bromide
EtOH	ethanol
GlcNAc	N-acetylglucosamine
GlcNAcK	N-acetylglucosamine kinase
HCl	hydrochloric acid
IPTG	iIsopropyl-β-D-thiogalactoside
Jacq.	Jacquin
kb	kilobase
KCl	potassium chloride
LB	Luria-Bertani
LiCl	lithium chloride
LSC	Liquid scintillation counter
M	molar
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MMLV	murine moloney leukemia virus
MOPS	3-(N-morpholino)propane-sulphonic acid
MPOB	Malaysian Palm Oil Board
Na_2CO_3	sodium carbonate
NAA	napthylacetic acid
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOAc	sodium acetate
NaOH	sodium hydroxide
NBT	Nitroblue tetrazolium chloride
NEC	Non-embryogenic calli

N-terminal	amino terminal
NTP	nucleotides triphosphate
ºC	degree centigrade
OD	optical density
ORF	open reading frame
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
Poly A^+ RNA	polyadenylated RNA
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
rRNA	ribosomal RNA
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
SAM	Shoot apical meristem
SA-PMPs	Streptavidin-paramagnetic particles
SC	Suspension cultures
SDS	sodium dodecyl sulphate
TAE	tris acetate EDTA
UV	ultraviolet
v/v	volume per volume

w/v	weight per volume
WAA	weeks after anthesis
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	alpha
β	beta
γ	gamma
λ	lambda
μ	micro
ρ	para
Mg	magnesium
Gly	glycine
Lys	lysine
Gln	glutamine
Asp	aspartic acid
Asn	asparagines
Arg	arginine
ATP	adenosine triphosphate
Tyr	tyrosine

CHAPTER 1

INTRODUCTION

There is an estimated ready market for more than a hundred million oil palm tissue culture plantlets in the world. The oil palm, *Elaeis guineensis* Jacq., is only amenable for vegetative propagation by means of somatic embryogenesis. However, the tissue culture process has posed several problems including the low embryogenesis rates and abnormalities arising from tissue culture. It is thus important to alleviate these problems to improve the production scale, labour usage, efficiency and cost effectiveness of the process to ultimately meet the increasing demands for oil palm materials.

As the molecular studies of oil palm embryogenesis is still relatively new, being initiated only at the end of the last decade, efforts are partly focused on finding potential molecular markers that will assist in differentiating the embryogenic callus from the non-embryogenic callus. This early identification would enable reductions in terms of time and costs in the tissue culture process.

In this study, a few approaches have been taken to identify these potential markers. One of which is through cold plaque screening (Hodge *et al.*, 1992), a technique that allows the isolation of medium and low abundant genes. It has been used previously in the isolation of low or medium abundant transcripts from various cDNA libraries (Ng *et al.*, 1996; Schmidt *et al.*, 1997; Frugier *et al.*, 1998). Most genes involved in the regulation of developmental pathways for example transcription factors are

normally present at very low amounts in the cell. Initial efforts are targeted on the isolation of putative low abundant genes from oil palm suspension cultures, one of the earliest stages in oil palm embryogenesis. The clones obtained will be further screened for their preliminary expressions in embryogenic compared with non-embryogenic callus. Subsequently, efforts can be focused on selected clones based on their preliminary expression profiles as well as from their sequence identities and functional inferences from the database.

A host of homeodomain proteins play important roles in vertebrates and invertebrate embryogenesis including *Drosophila*, mouse and humans. In the past decade, plant homeodomain proteins have been isolated and divided into various families, inclusive of the KN1 (KNOTTED1) and the HD-Zip (homeodomain-leucine zipper) families of proteins which have been found to be involved in the developmental functions of the plant as well as in the plant's responses to external environmental stimuli. The class 1 *knox* genes have been found to be expressed early in embryogenesis, mainly in the maintenance of the apical meristem regions (Mayer *et al.*, 1998; Long and Barton, 1998; Chan *et al.*, 1998). Another homeodomain protein, WUSCHEL, is expressed very early in embryogenesis and functions probably in the initiation and maintenance of stem cell fate in the apical meristem of the embryo (Mayer *et al.*, 1998). Thus, certain plant homeodomain proteins seems to have important roles in embryogenesis as well, however, whether their roles are as extensive as compared to animal embryogenesis remains to be determined.

The shoot apical meristem is established during embryogenesis and is crucial in the vegetative and reproductive development of the plant. As oil palm has a single

vegetative meristem, it is imperative that the apical meristem is properly formed as early as the embryogenic stage. Many genes have been found to be important in the establishment and maintenance of the shoot apical meristem from the embryogenic stage onwards, including particular homeodomain proteins, NAC-domain containing genes and the CLAVATA group of proteins. Certain members of the NAC-domain containing genes, the *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUP-SHAPED COTYLEDON2* (*CUC2*) activate *SHOOT MERISTEMLESS* (*STM*) either directly or indirectly (Aida *et al.*, 1999). *STM*, a class 1 *knox* gene is involved in the initiation and maintenance of the shoot apical meristem. *CUC1* and *CUC2* also act redundantly to mark the boundaries of developing cotyledon primordia and floral organ primodia.

Hence, efforts were also undertaken to isolate some of these genes from oil palm embryogenic cultures that may have significantly similar roles in oil palm embryogenesis as well. Subsequently, characterization studies would be carried out on these genes of interest.