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USE OF REPRESENTATIONAL DIFFERENCE ANALYSIS TO REVEAL DIFFERENCES BETWEEN TRUNCATED LEAF SYNDROME AND NORMAL OIL PALM RAMET

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By

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Truncated Leaf Syndrome (TLS) is a commonly found abnormality amongst tissue cultured plantlets of oil palm (*Elaies guineensis* Jacq.) which, if severe, will eventually lead to the death of the ramets. It was hypothesized that this phenotype could be due to genetic or epigenetic variability. The first part of this study was aimed to identify the genetic variability via Genomic-Representational Difference Analysis (G-RDA), a technique whereby the differences between two closely related genomes can be identified. In this part, 2 clones of oil palm ramets (1181; severe vs normal and 2751; mild vs normal) were used as starting material with 4 rounds of forward and reverse G-RDA were performed. A total of 18 unique sequences from G-RDA were successfully obtained. Primers were designed and verification of forward G-RDA products through PCR analyses and sequence comparison was carried out using 12 clones of TLS and normal oil palm ramets. Two out of 18 set of primers [F4(6)-1181Bgl and F4(10)-1181Bgl] were identified as potential markers and further verified by PCR and Southern analyses. The primer set F4(6)-1181Bgl was only able to distinguish between TLS and normal ramets of only one genotype (Yangambi) with the presence of expected band in TLS but was absent in normal ramets. The primer set F4(10)-1181Bgl showed the presence of multiple banding pattern in the genotype of La Me and Yangambi. Analysis of the multiple bands sequences revealed that those sequences represent multiple regions within the same genome, hence it is potentially a polymorphic marker. The 2 primer sets mentioned above could be classified as potential genotype specific primers as it is only functional in selected genotype. The second part of this study was aimed to identify the epigenetic variability via Methylation Sensitive-Representational Difference Analysis (MS-RDA). In this part, 2 rounds of forward and reverse MS-RDA were carried out and a total of 4 differentially methylated sequences with matches to known gene were successfully obtained. Primers were designed based on the 4 target genes namely protein ycf68, 3-ketoacyl-CoA synthase 12, Scarecrow-like protein 9 and Nucleotide-diphospho-sugar transferases superfamily protein and verification process was carried out by Quantitative-PCR to identify the respected expression level in normal and TLS ramets. The relative expression level of uncharacterized protein ycf68 is up-regulated, while 3-

ketoacyl-CoA synthase 12, Scarecrow-like protein 9 and Nucleotide-diphospho-sugar transferases superfamily protein were down-regulated in TLS ramets as compared to the normal ramets. Further verification with extensive number of samples is needed to elucidate the potential of the above 2 primer sets from G-RDA and 4 primer sets from MS-RDA to be used as markers across all genotypes of oil palm.

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

PENGGUNAAN ANALISIS PERBEZAAN PERWAKILAN UNTUK MENGENALPASTI PERBEZAAN ANTARA ANAK POKOK KELAPA SAWIT YANG MENGALAMI SINDROM DAUN TERBANTUT DAN NORMAL

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Sindrom daun terpangkas (TLS) adalah ketidaknormalan yang biasa ditemui di kalangan anak pokok tisu kultur daripada kelapa sawit (*Elaies guineensis* Jacq.) yang mana, jika teruk, akhirnya akan membawa kepada kematian anak pokok tersebut. Ia telah dihipotesiskan bahawa fenotip ini mungkin disebabkan oleh perubahan genetik atau epigenetik. Bahagian pertama kajian ini bertujuan untuk mengenal pasti perubahan genetik melalui teknik genomik-analisis perbezaan perwakilan (G-RDA), teknik di mana perbezaan antara dua genom berkait rapat dapat dikenal pasti. Dalam bahagian ini 2 jenis klon anak pokok kelapa sawit (1181; parah vs normal dan 2751; kurang parah vs normal) telah digunakan sebagai bahan permulaan dengan 4 pusingan G-RDA ke hadapan dan ke belakang telah dijalankan. Sebanyak 18 jujukan unik daripada G-RDA telah berjaya diperolehi. Pencetus telah direka dan verifikasi produk G-RDA melalui analisis PCR dan perbandingan jujukan telah dilaksanakan dengan menggunakan 12 klon kelapa sawit yang TLS dan normal. Dua daripada 18 set pencetus [F4 (6) -1181Bgl dan F4 (10) -1181Bgl] telah dikenal pasti sebagai penanda molekular berpotensi dan seterusnya disahkan dengan PCR dan analisis Southern. Set primer F4 (6) -1181Bgl hanya dapat membezakan satu genotip (Yangambi) ramets TLS dan normal dengan kehadiran jalur di ramet TLS tetapi tidak di ramet normal. Set primer F4 (10) -1181Bgl menunjukkan kehadiran beberapa corak jalur dalam genotip La Me dan Yangambi. Analisis kehadiran beberapa corak jalur tersebut mendedahkan bahawa jalur-jalur tersebut mewakili pelbagai kawasan di dalam genom yang sama yang boleh dikatakan penanda berpotensi polimorfik. Dua set pencetus yang dinyatakan di atas boleh diklasifikasikan sebagai pencetus genotip berpotensi tertentu kerana ia hanya berfungsi dalam genotip tertentu sahaja. Bahagian kedua kajian ini bertujuan untuk mengenal pasti kepelbagaian epigenetik melalui teknik metilasi sensitif-analisis perbezaan perwakilan (MS-RDA). Dalam bahagian ini, 2 pusingan MS-RDA ke hadapan dan ke belakang telah dijalankan dan sebanyak 4 jujukan bermetil yang menunjukkan padanan dengan gen sasaran telah berjaya diperolehi. Empat set primer telah direka berdasarkan 4 sasaran gen iaitu "uncharacterized proteiun ycf68" adalah diekspresikan sedikit, manakala "3-ketoacyl-CoA synthase 12", "Scarecrow-like protein 9" dan "Nucleotide-diphospho-sugar

transferases superfamily protein" dan pengesahan telah dilakukan dengan menggunakan kaedah kuantitatif-PCR untuk mengenal pasti tahap ekspresi jujukan-jujukan tersebut dalam klon TLS dan normal. Tahap ungkapan relatif "uncharacterized proteiun ycf68" adalah diekspresikan sedikit, manakala "3-ketoacyl-CoA synthase 12", "Scarecrow-like protein 9" dan "Nucleotide-diphospho-sugar transferases superfamily protein" adalah diekspresikan banyak di klon TLS berbandingan dengan klon normal. Pengesahan lanjutan dengan jumlah samel yang banyak diperlukan untuk menjelaskan potensi dua set primer peroleh daripada G-RDA dan 4 set primer yang diperoleh daripada MS-RDA untuk digunakan sebagai marker di semua genotip kelapa sawit.

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I certify that a Thesis Examination Committee has met on 20 November 2015 to conduct the final examination of Kanagamalar Silvarajoo on her thesis entitled "Use of Representational Difference Analysis to Reveal Differences between Truncated Leaf Syndrome and Normal Oil Palm Ramet" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the (insert the name of relevant degree).

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius
µg	microgram
µl	microliter
µM	micromolar
♀	female
♂	male
2,4-D	2,4-dichlorophenoxyacetic acid
A	adenosine
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
C	cytidine
cDNA	complementary DNA
CTAB	cetyl trimethylammonium bromide
dATP	2'-deoxy-adenosine-5'-triphosphates
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC-dH_2O	diethylpyrocarbonate treated water
dGTP	2'-deoxy-guanisine-5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides triphosphates
dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	2'-deoxy-thymidine-5'-triphosphate
dUTP	2´-deoxyuridine, 5´-triphosphate
EDTA	ethylene diaminetetra acetic acid disodium salt
EEPS	N-(2-hydroxyethyl) piperazine-N-3-ropanesulfonic acid
E-value	expect value
FELCRA	federal land consolidation and rehabilitation authority
FELDA	federal land development authority agriculture services sdn. bhd.
g	gram
g	gravitational acceleration
G	guanosine
GC content	guanine-cytosine content
G-RDA	genomic representational difference analysis
IPTG	isopropyl β-D-1-thiogalactopyranoside
ISSR	inter-simple sequence repeat
Jacq.	Jacquin
kb	kilo base pair
KCl	potassium chloride
LB	Luria Bertani
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
ml	millilitre
mM	milimolar

MOPS	3-morpholinopropane-1-sulfonic acid
MPOB	malaysian palm oil board
MS-RDA	methylation sensitive representational difference analysis
N	normality
Na_2HPO_4	disodium phosphate
NAA	α-napthalene acetic acid
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCBI	national centre for biotechnology information
ng	nanogram
nm	nanometer
ORF	open reading frame
PCR	polymerase chain reaction
PVPP	polyvinylpolypyrrolidone
Q-PCR	quantitative PCR
RAPD	randomly amplified polymorphic DNA
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SNPs	single nucleotide polymorphisms
SSC	sodium chloride sodium citrate buffer
ssDNA	single stranded DNA
SSR	simple sequence repeat
T	thymidine
TAE	tris-acetate-EDTA
TE	tris-EDTA
TLS	truncated leaf syndrome
Tm	melting temperature
Tris-Cl	tris-chloride
Tris-HCl	tris-hydrochloric acid
tRNA	transfer RNA
U	unit
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
β	beta

CHAPTER 1

INTRODUCTION

Elaies guineensis, known as African oil palm or macaw-fat native to west and southeast Africa, is a monocotyledonous plant belonging to the Aracaceace family. It is the most important and valuable palm species which is mainly used in commercial agriculture for the production of palm oil and palm kernel oil. It is also known as an important food that serves as a major source of vegetable oils and fats/lipids. The palm oil and palm kernel oil is derived from fleshy mesocarp and hard kernel of oil palm fruit respectively. The palm oil is normally utilized in food industries whereas palm kernel oil is utilized in oleochemical industries for soaps, detergents and toiletry products making. The African palm is mainly propagated in Asia, particularly in Malaysia and Indonesia.

In Malaysia, it was reported that throughout the year of 2013 the production of palm oil is 19.21 million tonnes per hectare and the exports reached 18.15 million tonnes with the earning of RM 45, 269.23 million has been recorded (MPOB, 2014). In the year of 2014, the production of palm oil is 19.67 million tonnes per hectare with an increase of 2.29 % compared to the previous year. The export of palm oil in 2014 reached 17.31 million tonnes earning RM 44, 498.45 million (MPOB, 2015). As for the year of 2015, the production of palm oil is 7.28 million tonnes per hectare and the exports reached 6.17 million tonnes with an earning of RM 14, 623.40 from January – May 2015 (MPOB, 2015). The demand for palm oil as a major source of vegetable oil increases every year and is assumed to reach up to 240 million tonnes by the year of 2050 (Corley, 2009). To maintain as one of the most important producer and exporter of palm oil, Malaysia tries to meet the future demands by producing clonal palms through biotechnology approaches.

The propagation by tissue culture was first described in the 1970's (Jones, 1974). Since then, the commercial advantage of tissue culture planting materials over conventional seedlings of oil palm has been well established (Sogeke, 1998). The whole process that involves the production of oil palm via tissue culture from initial level to the development of the matured oil palm tree and also including the testing part takes about 10 years. Tissue culture method enables the replication of individual high yielding oil palm in large scale in shorter time compared to the conventional method. Tissue culture usually produces offsprings identical to that of the original palm and desired trait such as higher oil or bunch is highly heritable and transferable to the next generation with slow vertical growth and disease resistance (Mutert et al, 1999). It has been proven that clonal palms are able to increase the oil yield compared to the commercial palms (Sharifah and Abu, 2007; Khaw and Ng, 1997; Donough and Lee, 1993).

Unfortunately, the tissue culture process sometimes generates somaclonal variants or abnormality in oil palm known as Truncated Leaf Syndrome (TLS). The mean frequency of this abnormality occurrence in a clonal palm is approximately 20 %. This abnormality is only obvious after few weeks of the plantlets are transferred from the

culture media to the nursery. The TLS ramets will show different characteristics compared to the normal ramets. The TLS ramets had stunted growth as well as produced undeveloped roots and the leaves looked like grasshopper damage which will eventually lead to the death of the TLS ramets. It is not known why only some ramets in an oil palm clone become TLS while others produce normal ramets. It is hypothesized that the presence of such phenotype could be due to genetic changes at the genomic level or epigenetic variability namely altered methylation level.

As such, the specific objectives of this study were to compare and identify possible genomic differences between TLS and normal oil palm ramets using the Genomic Representational Difference Analysis (G-RDA) method and to compare and identify possible methylation differences between TLS and normal oil palm ramets using the Methylation Sensitive Representational Difference Analysis (MS-RDA).

This study was specifically carried out to identify genetic and methylation differences associated to the TLS ramets. The identification of such differences could serve as a marker to screen for the TLS ramets at the early stage of the oil palm tissue culture process. For this study, two different approaches were carried out to identify the differences between TLS and normal ramets. The subtraction of the TLS genome with the genome of normal ramet was performed to identify the differences in the genomic fragments using the G-RDA and MS-RDA approach. Selected oil palm sequences from G-RDA were subjected to Polymerase Chain Reaction (PCR) analysis, sequencing and southern analysis while those sequences selected from MS-RDA were investigated on its expression profile using Real-Time PCR analysis.

The genomic fragments isolated from G-RDA and MS-RDA approach may correspond to the genetic or epigenetic changes that might cause the differences between the genome of TLS and normal ramets. Therefore, these fragments have the potential to be used as molecular markers for differentiating the TLS ramets from normal ramets in the early stage of oil palm tissue culture process. The detection of genetic or epigenetic differences between TLS and normal oil palm ramets could improve the oil palm tissue culture efficiency which will reduce cost, labour and time. Once a promising molecular marker has been established, oil palm tissue culture becomes efficient in producing economically valuable clonal palm that eventually increases the yield of palm oil.

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