UNIVERSITI PUTRA MALAYSIA

REPRESENTATIONAL DIFFERENCE ANALYSIS TO IDENTIFY GENOMIC DIFFERENCES BETWEEN *Musa acuminata x balbisiana* cv Mutiara AND *Musa acuminata x balbisiana* cv Rastali IN RELATION TO FUSARIUM TOLERANCE.

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By

YONG SOCK HWA

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the degree of Master of Science.

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September 2009

Chair: Suhaimi Napis, PhD

Faculty: Falcuty of Biotechnology and Biomolecular Sciences

Inherited characteristics of an organism are the reflections of gene structure and organization, including interactions among different genes and their products, as well as environmental factors. Thus, variations in other genes may affect the expression or activity of proteins encoded by specific genes. The study of genetic variation is essential in order to examine differences among members of the same species, such as to differentiate between individuals. *Musa acuminata x balbisiana* cv Rastali is very susceptible to Fusarium Wilt Race 1 and Race 4, which is caused by the soil inhibiting fungus known as *Fusarium oxyporum* f. sp. *Cubense*. Micropropagation of Fusarium wilt tolerant *M. acuminata x balbisiana* cv Rastali selection has been successfully developed by United Plantations Bhd. These bananas have shown a high tolerance to Fusarium wilt race 4 and it is known as *M. acuminata x balbisiana* cv Mutiara. Fusarium wilt tolerant *M. acuminata x balbisiana* cv Rastali selection is based on the screening of banana clones

by field testing in the “Fusarium Hot-Spot”. The process is time consuming and the process may result in a disease outbreak. Thus, polymorphic markers to differentiate *M. acuminata x balbisiana* cv Rastali from *M. acuminata x balbisiana* cv Mutiara will facilitate the early identification and screening process. In this study, representational difference analysis (RDA) approach has been used to identify genomic differences between *M. acuminata x balbisiana* cv Mutiara and *M. acuminata x balbisiana* cv Rastali. A total of 56 difference products were isolated from the variable sequences present in the genomes of both cultivars with two enzymes and four subtractions. These clones were selected for sequencing and homology search against the available databases. Generally, the two cultivars showed a high degree of genomic similarity (identities > 98 %). Base changes and short deletions of DNA sequences of both cultivars were detected by the sequence analysis of 8 interesting clones that were expressed during the infection of *M. acuminata x balbisiana* cv Mutiara. One of the prospective clone, 1.2-5b is homologous to chitinase class III which is a plant defence related gene. It was found to only express in the root of infected *M. acuminata x balbisiana* cv Mutiara although it was present in both *M. acuminata x balbisiana* cv Mutiara and *M. acuminata x balbisiana* cv Rastali genomic DNA. The amplified DNA fragment using primer 1.2-5b for both cultivars is different in length. The most variable region was found at the sequence after the specific forward primer site and the identity of sequence was found at the second half of the sequence. Besides, primer 2.2-9 also gave a different amplified product size, whereby the amplified fragment of *M. acuminata x balbisiana* cv Mutiara is 25 bp longer than the *M. acuminata x*

balbisiana cv Rastali. Homology search of the sequence from *M. acuminata x balbisiana* cv Mutiara was unknown. Both cultivars can be easily distinguished from each other by using primers 1.2-5b and 2.2-9. The highly variable region in the sequence of 1.2-5b and 2.2-9 for both cultivar might be due to the natural mutation and environment stresses for the *M. acuminata x balbisiana* cv Mutiara which derived from *M. acuminata x balbisiana* cv Rastali to be Fusarium tolerance and survived. The RDA approach has successfully isolated and identified potential variable regions of DNA fragments that might be related to their genotype as Fusarium wilt tolerant and Fusarium wilt susceptible cultivars.

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

ANALISIS PERWAKILAN PERBEZAAN UNTUK MENGENALPASTI PERBEZAAN GENOMIK ANTARA *Musa acuminata x balbisiana* cv Mutiara DAN *Musa acuminata x balbisiana* cv Rastali DENGAN HUNGBUNGKAIT RINTANGAN TERHADAP FUSARIUM..

Oleh

YONG SOCK HWA

September 2009

Pengerusi: Suhaimi Napis, PhD

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Ciri- ciri yang diwarisi oleh sesuatu organisma adalah hasil daripada struktur dan organisasi gen. Ini termasuk interaksi antara gen-gen yang berlainan dan produknya, serta faktor-faktor persekitaran. Maka, variasi pada gen-gen lain boleh mempengaruhi pengekpresan atau aktiviti protein-protein yang dikodkan oleh gen-gen tertentu. Penyelidikan dalam variasi genetik memainkan peranan yang penting dalam membolehkan kajian pembezaan antara individu daripada spesis yang sama. *M. acuminata x balbisiana* cv Rastali didapati mudah dijangkiti oleh Fusarium Wilt Race 1 and Race 4, yang disebabkan oleh kulat perencat tanah (soil inhibiting fungus) iaitu *Fusarium oxyporum* f. sp. *Cubense*. Melalui mikropropagasi *M. acuminata x balbisiana* cv Rastali yang rintang terhadap penyakit layu Fusarium telah berjaya dihasilkan oleh United Plantations Bhd. Pisang-pisang ini telah menunjukkan tahap kerintangan yang tinggi terhadap penyakit layu Fusarium ras 4 dan pisang ini dikenali sebagai *M. acuminata x balbisiana* cv Mutiara.

Pemilihan pisang ini dilakukan semasa ujian tapak klon-klon pisang yang berada di “Fusarium Hot-Spot”. Namun demikian, proses ini memakan masa yang lama dan ia berkemungkinan menyebabkan penyebaran penyakit. Maka, dengan menggunakan penanda polimorfik dalam proses penyaringan, ia dapat memudahkan proses pembezakan antara *M. acuminata x balbisiana* cv Rastali dan *M. acuminata x balbisiana* cv Mutiara. Dalam penyelidikan ini, pendekatan analisis perwakilan perbezaan (RDA) telah digunakan untuk mengenalpasti perbezaan genomik antara *M. acuminata x balbisiana* cv Mutiara dan *M. acuminata x balbisiana* cv Rastali. Dengan menggunakan dua enzim dan empat penolakkan (“Subtraction”), sebanyak 56 produk bezaan (“difference products”) telah dipencilkan hasil daripada variasi jujukan yang hadir dalam genom kedua-dua kultivar. Klon-klon ini telah dipilih bagi tujuan penjujukan dan pencarian homologi dengan pangkalan data yang sedia ada. Salah satu klon yang berpotensi iaitu 1.2-5b adalah homolog kepada kitinase kelas III. Kitinase kelas III merupakan gen yang berkaitan dengan daya pertahanan tumbuhan. Ia didapati hanya diekpresikan dalam akar *M. acuminata x balbisiana* cv Mutiara yang dijangkit penyakit Fusarium walaupun gen ini hadir dalam kedua-dua genomik DNA *M. acuminata x balbisiana* cv Mutiara and *M. acuminata x balbisiana* cv Rastali. Keratan DNA yang diamplifikasi daripada kedua-dua kultivar dengan menggunakan pencetus 1.2-5b adalah berlainan saiznya. Bahagian jujukan yang paling berbeza adalah di bahagian jujukan DNA selepas pencetus depan. Manakala, jujukan DNA di bahagian belakang keratan DNA ini adalah sama. Selain itu, keratan DNA klon 2.2-9 juga memberikan saiz produk amplifikasi yang berbeza, di mana produk amplifikasi *M. acuminata x balbisiana* cv

Mutiara adalah 25 pb lebih panjang berbanding dengan M. acuminata x balbisiana cv Rastali. Keputusan homologi menunjukkan jujukan DNA tersebut adalah tidak dapat dikenalpasti ("unknown"). Kedua-dua kultivar boleh dibezakan dengan menggunakan pencetus 1.2-5b and 2.2-9. Bahagian jujukan DNA yang berbeza ini mungkin disebabkan oleh mutasi semula jadi dan factor tekanan persekitaran bagi *M. acuminata x balbisiana* cv Mutiara yang berasal usul daripada *M. acuminata x balbisiana* cv Rastali yang rintang terhadap Fusarium dan berupaya terus hidup. Pendekatan RDA telah berjaya memencil dan mengenalpasti rantau variasi genomik DNA yang mungkin berkaitan dengan perbezaan genetik kultivar yang rintang terhadap penyakit layu Fusarium dan kultivar yang tiada rintangan terhadap penyakit layu Fusarium.

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I certify that a Thesis Examination Committee has met on 16 September 2009 to conduct the final examination of Yong Sock Hwa on her thesis entitled "Representational Difference Analysis to Identify Genomic differences between *Musa acuminata x balbisiana* cv Mutiara and *Musa acuminata x balbisiana* cv Rastali in Ralation to Fusarium Tolerance" in accordance with Universties 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

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

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Date: 14 January 2010

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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YONG SOCK HWA

Date: 16 September 2009

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LIST OF ABBREVIATIONS

β	beta
µg	microgramme
µl	microliter
℃	degree centrigrade
%	percentage
AFLP	Amplified fragment length polymorphism
Amp	ampicillin
bp	base pair
BLAST	basic local alignment search tool
BSA	bovine Serum Albumin
cm	centimeter
DNA	deoxyribonucleic acid
cDNA	complementary DNA
CTAB	Cetyltrimethylammonium Bromide
dNTPs	deoxynucleotides
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dTTP	thymidine-5'-tryphosphate
DEPC	dietyl pyrocarbonate
DPs	differential products

DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EPPS	N-2-hydroxyethylpiperazine-N-3-propane sulfonic acid
Foc	*Fusarium oxysporum* f. sp. *cubense*
g	gramme
hr	hour
IRAP	Inter-retrotransposon amplified polymorphism
kb	kilobase-pair
LB	luria-bertani
LiCl	lithium chloride
M	molar
mg	milligram
min	minute(s)
mm	millimeter
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	nanogramme
OD	optical density
PCR	polymerase chain reaction

PVP	polyvinylpyrrolidone
RAPD	Random amplified polymorphic DNA
RDA	Representational Difference Analysis
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
SDS	sodium dodecyl sulphate
SSC	sodium chloride-sodium citrate buffer
TAE	tris acetate EDTA
TE	tris-HCl-EDTA
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Bananas are the developing world's fourth most important food crop after rice, wheat and maize. It is a major table fruit worldwide. They are easily grown and do not need to be replanted. Bananas, especially the cooking varieties that are boiled, steamed, fried or roasted, provide an essential staple food for more than 100 million people in Africa. Besides their nutritional value, bananas have a soothing effect in the treatment of gastric ulcers and diarrhea. They are also a good source of energy, rich in vitamins A, C and B6 and contain high levels of several minerals, such as calcium, potassium and phosphorus.

Banana is the most common fruit in Malaysia and it is chiefly eaten raw or fried as a dessert fruit. In Malaysia, the popular dessert cultivars are Pisang Mas, Pisang Berangan, Pisang Rastali, and Pisang Embun while the popular cooking cultivars are Pisang Raja, Pisang Nangka, Pisang Tanduk, Pisang Awak, and Pisang Abu Nipah. Despite increasing demand, banana production has decreased in the country due to disease, high labour costs and marketing and distribution problems. One of the most serious threats is Fusarium wilt or Panama disease, which is caused by the soil-borne fungus *Fusarium oxysporum formae specialis (f. sp.) cubense* (Foc) (Peraza-Echeverria *et al*., 2008). This fungal pathogen will remain in the infected field for 30 years or more

(Moore *et.al.*, 1995; Stover, 1962). There is no chemical control available. The only way to control it is to utilize cultivars that are resistant or tolerant to the pathogen (Zambrano *et al.*, 2007). Most of the local banana varieties in Malaysia, such as *M. acuminata x balbisiana* cv Rastali, local name as Pisang Rastali, are susceptible to the disease. However, *M. acuminata x balbisiana* cv Mutiara (Pisang Mutiara), which is an improved cultivar of *M. acuminata x balbisiana* cv Rastali has shown high tolerance to Fusarium wilt. Both *M. acuminata x balbisiana* cv Rastali and *M. acuminata x balbisiana* cv Mutiara are AAB cultivars. Fusarium wilt tolerance *M. acuminata x balbisiana* cv Mutiara (Pisang Mutiara) is the selected surviving micropropagated clones challenged in "Fusarium Hot-Spot" field (Ho, 1998).

Selection of clones from the infected field is time consuming and labor-intensive. Besides, it has a high risk of spreading the pathogen to other field. Thus, genomic differences between these two highly similar genomes are important to gain an insight for tolerance and susceptibility of the banana which may lead to a more efficient method of identifying Fusarium wilt tolerance *M. acuminata x balbisiana* cv Mutiara (Pisang Mutiara).

Representational difference analysis (RDA) is one of the approaches to find genomic differences between two closely related genomes and was developed by Lisitsyn and Wigler (1993). It has been used to identify probes for any genomic deletions, rearrangements, insertions, amplifications, or point

mutations between two complex genomes that result in appropriate restriction fragment length polymorphism (RFLP) (Lisitsyn and Wigler, 1993).

Thus, the objective of this study is to utilize RDA approach to identify genomic differences between *M. acuminata x balbisiana* cv Mutiara and *M. acuminata x balbisiana* cv Rastali. The specifics objectives are:

(1) To isolate and identify genomic differences between *M. acuminata x balbisiana* cv Mutiara and *M. acuminata x balbisiana* cv Rastali using RDA approaches.

(2) To carry out comparative analysis of the sequences produced by this subtraction method from both cultivars.