UNIVERSITI PUTRA MALAYSIA

BIOCHEMICAL AND MOLECULAR STUDIES OF ACTIVE AND PASSIVE DEFENSE SYSTEMS IN *MUSA ACUMINATA* L. CV. 'JARI BUAYA'

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BIOCHEMICAL AND MOLECULAR STUDIES OF ACTIVE AND PASSIVE DEFENSE SYSTEMS IN *MUSA ACUMINATA* L. CV. 'JARI BUAYA'

By

WAY CHIANG POH

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

September 2006

DEDICATED TO:

BELOVED A-MA, BROTHER AND SISTERS, ONG KOK ENG,

ALSO NOT MISSING THE LECTURERS AND FRIENDS.

WHO ALWAYS HAVE CONFIDENCE IN ME. THEIR FAITH AND ENDLESS SUPPORTS AS MY STRENTH PILLARS THAT DIRECTING ME GONE THROUGH ALL THE OBSTACLES IN THE LIFE.

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

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Chairman: **Mohd. Puad Abdullah, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Fusarium wilt incidence constitutes one of the major constraints in the development of sustainable banana industry in the country. To date, a cost-effective measure of control for this disease is still not available and farmers are still depending heavily on the use of chemicals to minimize the problem. The use of resistant cultivar to enhance resistant to critical diseases and the reduction of chemicals usage holds the key to surge a better profit margin in the industry. The approach employed in this study was to enhance disease resistant of some of the existing banana cultivars in Malaysia by using biotechnology technique. The objectives of this study were isolation of genes related to plant defense system by using PCR, and analyses of resistant gene candidates (RGCs) in specific host-pathogen interaction, by using reverse transcription-polymerase chain reaction (RT-PCR). Five out of six of the putative diseases RGCs were differentially expressed in the 'Jari Buaya' (JB)- *Fusarium oxysporum* cubense race 4 (Focr4) interactions. RGC1 was induced and involved in this interaction. In addition, the

expression of RGC1 was not affected water stress. The transcripts level of RGC2 and RGC3 decreased as the infection progressed from 1 hour to 3 hours. The mentioned three different RGCs belong to the class of nucleotide binding site-leucine rich repeat (NBS-LRR) disease related proteins. Southern hybridization analyses depicted that these genes belonged to a small gene family. The other three RGCs, RGC4, RGC5 and RGC6 were classified in the kinase family. RGC4 and RGC5 were constitutively expressed in JB-Focr4 interaction and no expression was observed for RGC6. This is the first report of globally renowned Focr4 resistant of local banana crop 'Jari Buaya' at molecular level in plant active defense system.

The accumulation of phenolic compounds and often lignin in plant tissues especially the cell wall and vascular system, is an established plant response to fungal attack. This response has been hypothesized to play an important role in determining passive resistant. Focr4 resistant cultivar, 'Jari Buaya' possessed higher amount of phenolic compounds for both the intracellular and cell wall. However, the susceptible cultivar, 'Rastali' secreted more phenolics than the resistant cultivar into the environment. The results were in tandem with the phenylalanine ammonia lyase (PAL) enzyme activity. Lignin staining of banana roots unveiled that the resistant cultivar possessed higher PAL activity and more lignin deposition in the roots than the susceptible cultivar.

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

KAJIAN BIOKIMIA DAN MOLEKUL SISTEM PERTAHANAN AKTIF DAN PASIF BAGI *MUSA ACUMINATA* L. CV. 'JARI BUAYA'

Oleh

WAY CHIANG POH

September 2006

Pengerusi: **Mohd. Puad Abdullah, PhD**

Fakulti: **Bioteknologi and Sains Biomolekul**

Kejadian kelayuan Fusarium merupakan salah satu daripada pengekang utama dalam pembangunan industri pisang negara. Pada masa ini, kaedah pengawalan berkesan masih belum ada dan petani masih banyak bergantung kepada penggunaan bahan kimia untuk mengurangkan masalah ini. Penggunaan kultivar pisang yang tahan kepada penyakit-penyakit yang kritikal dan pengurangan penggunaan bahan-bahan kimia menjadi kunci kepada peningkatan keuntungan dalam industri ini. Pendekatan kita adalah dengan penggunaan teknik-teknik bioteknologi untuk meningkatkan ketahanan penyakit kultivar pisang yang sedia ada. Projek ini bertujuan untuk memencilkan gen-gen yang berkaitan dengan sistem pertahanan tumbuhan dengan menggunakan kaedah PCR bagi tujuan pengawalan penyakit dengan mengenalpasti calon gen ketahanan (RGC) yang diekspres dalam kajian pengekspresan gen secara 'RT-PCR' melibatkan interaksi perumah-patogen yang spesifik. Lima daripada enam calon gen ketahanan telah menampilkan corak ekspresi yang berlanian dalam interaksi Jari Buaya-*Fusarium oxysporum* (Focr4).

RGC1 diaruh dan kemungkinan besar terlibat dalam interaksi ini. Tambahan pula, pengekspresan RGC1 tidak dipengaruhi oleh tekanan air. Akan tetapi, bilangan transkrip untuk RGC2 dan RGC3 berkurangan setelah jangkitan berlanjutan dari satu jam ke tiga jam. Ketiga-tiga gen tersebut digolongkan dalam kumpulan protein berkaitan penyakit tapak pengikatan nukleotida-kaya leusina berulang (NBS-LRR). Analisis 'Southern' menunjukkan protein kumpulan ini adalah dalam famili protein kecil. Tiga gen lagi dikategorikan dalam kumpulan 'kinase'. RGC4 dan RGC5 diekspreskan sepanjang masa dalam interaksi ini dan tidak ada ekspresi diperlihatkan untuk RGC6. Ini merupakan laporan pertama yang menampilkan pengajian di peringkat biologi molekul yang melibatkan sistem pertahanan aktif pisang Jari Buaya yang diketahui umum sebagai rintang kepada Focr4.

Pengumpulan sebatian yang lazimnya fenol dalam tisu tumbuhan terutamanya di dinding sel dan sistem vaskular merupakan reaksi tumbuhan terhadap serangan kulat yang telah diketahui, dan ia telah dihipotesis memainkan peranan penting dalam sistem pertahanan pasif tumbuhan kultivar rintang Focr4, Jari Buaya mempunyai kandungan sebatian fenol yang tinggi untuk kedua-dua jenis, intrasel dan yang terikat pada dinding sel. Bagaimanapun, Rastali ialah kultivar rentan Focr4 merembes lebih banyak sebatian fenol berbanding kultivar rintang Focr4. Keputusan tersebut selari dengan kajian enzim fenilalanina amonia liase (PAL) dan kajian histologi pewarnaan lignin yang menunjukkan kultivar rintang mempunyai aktiviti PAL yang tinggi dan memendapkan lebih banyak lignin pada tisu akar berbanding kultivar rentan.

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I certify that an Examination Committee has met on 26th September 2006 to conduct the final examination of Way Chiang Poh on his Master of Science thesis entitled "Biochemical, and Molecular Studies of Active and Passive Defense Systems in *Musa acuminate* L. CV. 'Jari Buaya' in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis 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 are as follows:

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Date: 14th DECEMBER 2006

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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WAY CHIANG POH

Date: 7th NOVEMBER 2006

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LIST OF ABBREVIATIONS

BAC	Baterial artificial chromosomes
bp	basepair
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CC	Coiled-coil
Ct	Cortex
CTAB	hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	deoxynocitinamide triphosphate
Ed	Endodermis
EDTA	Ethylenediaminetetraacetic acid
Ex	Exodermis
FAA	Formalin / acetic acid / ethanol
Focr4	*Furasium oxysporum* cubense race 4
GAE	Gallic acid equivalents
HCl	Hydrochloric acid
HR	Hypersensitivity response
IPA	Isopropyl alcohol
IRD	Infected root density
JA	Jasmonic acid
kb	kilo basepair
LAR	Localized acquire resistant
LB	Luria-Bertani

LRR	Leucine-rich repeat
LZ	Leucine zipper
MAPKs	Mitogen-activated protein kinases
$MgCl_2$	Magnesium chloride
Min	Minute
MS	Murashige and Skoog
NBS	Nucleotide binding side
NLB	Nuclear lysis buffer
NLS	Nuclear localization signals
NO	Nitric oxide
PAL	Phenylalanine ammonia lyase
PDA	Potato dextrose agar
pK	Kinase plasmid
P-loop	Phosphate binding site
pNBS	Nucleotide binding site plasmid
pNEB205A	Cloning vector
PR	Pathogenesis-related
RFLP	Restriction fragment length polymorphism
RGCs	Resistant gene candidates
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistant

SDS	Sodium dodecyl sulphate
SP	Signal peptide
SSH	Supression subtraction hybridization
SUMO	Small ubiquitin related modifier
TIR	Toll and interleukin-1 receptor
TMS	Transmembrane
TMV	Tobacco mosaic virus
Tris-Cl	Tris-chloride
VIGS	Virus-induced gene silencing
Vs	Vascular system

CHAPTER I

INTRODUCTION

Banana is recognized as one of the important food sources in the tropics with global sales of five billion (US) dollars per annum (Jalil *et al.,* 2003). Malaysia has 50 types of banana cultivars that may serve as sources of genetic variability for agronomic and disease improvement programs (Jalil *et al.,* 2003). However, *Fusarium oxysporum* (Fo) that is the causative agent of Fusarium wilt disease in many banana species is considered a major problem to the banana industry in Malaysia and worldwide. The management of Fusarium wilt is fastidious and it contributes to a substantial proportion of the overall production cost (Lheureux *et al.,* 2003). To date, 4 races of pathogen have been identified: races 1, 2, 3 and 4. *F. oxysporum cubense* race 4 (Focr4) is the most destructive in Malaysia, infecting almost all banana cultivars especially 'Rastali' is the most susceptible one, except for a few such as 'Jari Buaya', and 'Intan'. (Jeger *et al*., 1996). The fungus infects the roots during early growth stages of banana plant and gradually colonizes the vascular system which leads to total collapse of the plant.

Today, an integrated disease management is employed in areas where Fusarium wilt is a major problem in order to minimize heavy usage of pesticides and chemicals. This approach relies on good agricultural practices with limited usage of chemicals, and enhances of banana to infection of Focr4 and other major diseases to produce a more sustainable banana industry in Malaysia. The development of a long term disease control strategy remains as the top priority. With the advances of plant genetic engineering technology, cultivars with good disease properties have been exploited by breeders to

develop new planting materials with enhanced resistant to selected plant pathogens including the major ones. This has been the most economical and effective alternative of combating the disease. However, genetic improvement of banana for resistant to Focr4 infection is very difficult using conventional breeding methods due to the complex polyploidy genetic composition of banana. Thus, a better understanding of banana genomic structure and molecular characterization of disease resistant genes are crucial and of high priority (Wang *et al.,* 1999). Studies on the interaction between host plant and pathogen at molecular level for nearly two decades have revealed a specific gene-for-gene interaction existed between the host and the pathogen. The resistant gene (*R*) protein from cultivar interacts with the avirulence gene (*avr*) protein from pathogen triggering a defense response in the plant to combat the pathogen. The genetic engineering of this specific disease resistant gene from cultivar to susceptible cultivar was widely used in many plant species such as tomato, tobacco, *Arabidopsis* to produce a disease tolerant or trait (Ellis *et al.,* 2000; Rommens and Kishore, 2000; Rivas *et al.,* 2004).

Here, similar approach was used to isolate a set of gene candidates (RGCs) from 'Jari Buaya' which has been recognized as a cultivar to Focr4 (Bink *et al.,* 1997), using degenerate primers targeting the nucleotide binding site (NBS) and kinase subdomain consensus domains of *R*-genes. This prompted the examination of gene expression of the RGCs with RT-PCR in 'Jari Buaya'-Focr4 interaction, to gain insights into gene expression of active plant defense mechanism. Passive defense (lignification) was speculated to be involved in plant defense response along with the active defense, thus, comparative studies on the fundamental of physiological, biochemical and cellular

aspects involving the (‘Jari Buaya’) and susceptible (‘Rastali’) banana cultivars were also carried out.

These overall objectives of this study were:

1. To identify the putative disease candidates (RGCs) from ‘Jari Buaya’ that may be involved in active defense for specific ‘Jari Buaya’-Focr4 interaction.
2. To study the biochemical properties in terms of total phenolic compounds, phenylalanine ammonia lyase (PAL) activity and lignin deposition in the root tissue of ‘Jari Buaya’ in comparison with ‘Rastali, in order to reflect its promising passive defense properties.

CHAPTER II

LITERATURE REVIEW

Banana 'Jari Buaya' Cultivar Genotype

Banana is one of the most important crops in tropical regions. Majority of the cultivars are highly sterile, polyploid and derived from two major species: *Musa acuminata* and *Musa balbisiana* (Baurens *et al.,* 1996). However, the genetic composition of cultivated *Musa* is complex. Their genetic composition is usually represented with the letter codes A and B, representing the two wild species, respectively. The cultivars can be diploid (AA, BB) or tetraploid (AAAB, AABB, ABBB) but majority are triploid (AAA, AAB, ABB) (Geering *et al.,* 2001). Banana 'Jari Buaya' cultivar, which belongs to the group of *Musa acuminata,* is the most promising due to its disease resistant properties (Binks *et al.,* 1997).

Resistant (*R*) Genes in Combating Disease Problems in Banana Industry

The management of disease and pest in banana plantations covers not only problems associated with fungi (Fusarium wilt), but also viruses (banana streak virus), bacteria (bacterial blight disease), nematodes and various environmental disorders. Several diseases cause significant losses in the production of banana industry such as the banana streak disease caused by banana streak virus, black sigatoka, Fusarium wilt caused by *Fusarium oxysporum* (Fo) and so on (Jeger *et al*., 1996). However, Fo, which is the