
**UNIVERSITI PUTRA MALAYSIA**

# ISOLATION AND CHARACTERIZATION OF PHENYLALANINE AMMONIA LYASE (PAL) AND OTHER DEFENCE RESPONSE GENES FROM OIL PALM (*ELAIES GUINEENSIS* JACQ.)

**HWANG SIAW SAN**



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**By**

**HWANG SIAW SAN**

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**Chairman: Tan Siang Hee, Ph.D.**

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Enhanced disease resistance and plant defense response against pathogen attack and environmental stresses have always been important targets of plant biotechnology. The activation of the defense response requires the recognition of an elicitor and the subsequent initiation of a signal transduction pathway, which then leads to the activation of defense genes and the production of phytoalexins. Plants respond to pathogen attack and environmental stimuli by activating a wide variety of defense reactions including transcriptional activation of genes involved in phenylpropanoid biosynthesis, accumulation of antimicrobial phytoalexins, and ethylene production.

Phenylpropanoid metabolism is a plant specific pathway that leads to the production of secondary metabolites including isoflavonoid phytoalexins, lignin, flavonoid pigments and UV protectants such as furanocoumarin. Here, we report the isolation of several different clones that are involved in the plant defense response by screening of oil palm zygotic embryo and suspension culture cDNA libraries. These clones include

phenylalanine ammonia- lyase (PAL) (4-4A, 11F1 and 12B1), S-adenosylmethionine synthetase I (Adomet synthetase I) (11A1), peroxidase (7A2), chitinase III (4A3), calmodulin (3E2), and beta-ketoacyl-CoA synthase (KCS) (3G1).

PAL and peroxidase are enzymes involved in the phenylpropanoid pathway and lignin biosynthesis. Adomet synthetase I is a precursor for ethylene biosynthesis in plants and also acts as a methylating agent in cells. Calmodulin is a calcium-binding molecule that is involved in the signal transduction pathway during the plant defense response. On the other hand, chitinase III is one of the pathogenesis-related (PR) proteins whereas KCS plays an important role in plant protection through the production of epicuticular waxes on the plant surface.

Northern blot analyses have demonstrated the differential expression of PAL and Adomet synthetase I in response to wounding, UV irradiation and ethephon treatments. In addition, expression of PAL and Chitinase III in different tissues has also been studied. Southern blot analyses demonstrated that PAL and calmodulin appear as a single copy gene in the oil palm genome. Two copies of KCS and two or more copies of Adomet synthetase I, chitinase III, and peroxidase have been detected in the oil palm genome. In these experiments, the combination of a PCR-based screening technique and plaque lift hybridisation has proved to be an effective method, especially for the isolation of a specific gene from a cDNA library. Besides, this method is also less time consuming and less laborious.

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

**PENGASINGAN DAN PENCIRIAN PHENYLALANINE AMMONIA LYASE (PAL) DAN GEN-GEN PERTAHANAN YANG LAIN DARI KELAPA SAWIT (*ELAIES GUINEENSIS* JACQ.)**

**Oleh**

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**Februari 2002**

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Peningkatan taraf pertahanan tumbuhan terhadap serangan penyakit and tekanan persekitaran sentiasa menjadi tumpuan kepada bioteknologi tumbuhan hari ini. Perangsangan reaksi pertahanan tumbuhan memerlukan penghasilan elisitor dan kehadiran satu proses signal transduksi yang akan membawa kepada ekspresi gen-gen pertahanan dan penghasilan 'phytoalexin'. Tumbuhan bertindak balas terhadap serangan patogen dan rangsangan persekitaran dengan menghasilkan reaksi pertahanan termasuk transkripsional gen yang terlibat dalam penghasilan 'phenylpropanoid', pengumpulan antimikrobial 'phytoalexin', dan penghasilan etilena.

Metabolisma phenylpropanoid adalah satu proses spesifik yang membawa kepada penghasilan produk asli tumbuhan termasuk 'isoflavonoid phytoalexin', lignin, pigmen flavonoid dan bahan lindungan UV seperti 'furanocoumarin'. Di sini, kami melaporkan pengasingan gen-gen yang bertanggungjawab ke atas tindak balas pertahanan tumbuhan dengan penyaringan koleksi cDNA daripada embrio zigotik dan kultur ampaian kelapa

sawit. Klon-klon ini termasuk phenylalanine ammonia-lyase (PAL), S-adenosylmethionine synthetase I (Adomet synthetase I), peroksidase, kitinase III, calmodulin, dan β-ketoacyl-CoA synthase (KCS).

PAL dan peroksidase adalah enzim yang terlibat dalam metabolisma 'phenylpropanoid' dan penghasilan lignin. Adomet synthetase I adalah prekursor kepada penghasilan etilena dan ia juga merupakan satu agen metilasi dalam sel. Selain itu, calmodulin adalah molekul yang terikat kepada kalsium yang terlibat dalam proses signal transduksi semasa tindak balas pertahanan tumbuhan. Di samping itu, kitinase III adalah salah satu daripada patogenesis protein, manakala KCS memainkan satu peranan penting dalam perlindungan tumbuhan melalui penghasilan lilin kutikel di permukaan tumbuhan.

'Northern blot' analisis menunjukkan ekspresi yang berlaninan untuk PAL dan Adomet synthetase I terhadap perlukaan dan radiasi UV. Selain itu, ekspresi PAL dan kitinase III dalam tisu yang berlainan juga diselidiki. 'Southern blot' analisis mendemonstrasikan bahawa PAL dan calmodulin berkemungkinan wujud sebagai gen tunggal dalam genomik kelapa sawit. Lebih daripada satu gen dijangkakan terdapat dalam genomik kelapa sawit untuk Adomet synthetase I, kitinase III, peroksidase, dan KCS. Dalam eksperimen ini, penggunaan teknik penyaringan koleksi cDNA melalui PCR dan 'plaque lift' hybridisasi telah dibuktikan sebagai satu kaedah yang efektif, terutamanya untuk pengasingan gen-gen spesifik. Selain itu, ia juga menjimatkan masa dan tenaga kerja.

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science

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## DECLARATION

I hereby declare that the thesis is based on my original work except for equations 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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(HWANG SIAW SAN)

Date:

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| $A_{260}$ | absorbance at 260 nm |
| 2-BE | ethylene glycol monobutyl ether |
| BLAST | Basic Local Alignment Research Tool |
| 4CH | cinnamic acid 4-hydroxylase |
| 4CL | 4-coumarate:CoA ligase |
| ACC synthase | 1-aminocyclopropane-1-carboxylic acid |
| ACP | acyl carrier protein |
| AOPP | L-α-aminooxy-β-phenylpropionic acid |
| Avr gene | avirulence gene |
| $C_2H_2$ | ethylene |
| CAD | cinnamyl-alcohol-dehydrogenase |
| CaM | calmodulin |
| CCR | cinnamoyl-CoA reductase |
| cDNA | complementary deoxyribonucleic acid |
| CHS | chalcone synthase |
| DEPC | diethyl pyrocarnonate |
| DNA | deoxyribonucleic acid |
| dNTPs | dioxynucleoside triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetic acid |
| EGTA | ethylene glycol-bis(β-aminoethylether) -*N,N,N',N*-tetraacetic acid |
| ERE | ethylene responsive element |

| | |
|---|---|
| FAE | fatty acid elongase |
| GTE | Glucose-Tris-EDTA |
| GTP | guanosine triphosphate |
| GUS | β-glucoronidase |
| hr | hour |
| HR | hypersensitive reaction |
| HRGP | hydroxyproline-rich glyprotein |
| Jacq. | Jacquin |
| KCl | potassium chloride |
| KCS | beta ketoacylCoA synthase |
| kb | kilobase |
| LAR | localized acquired resistance |
| LB | Luria-Bertani |
| LiCl | lithium chloride |
| LRRs | leucine rich repeats |
| M | Molar |
| MAP | mitogen activated protein |
| mg | milligram |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulfate |
| min | minute(s) |
| mM | millimolar |
| MOPS | 3-(N-morpholino) propanesulfonic acid |
| mRNA | messenger ribonucleic acid |

| | |
|---|---|
| MYB | myoblastosis |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| NCBI | National Center for Biotechnology Information |
| ng | nanogram |
| OD | Optical density |
| oligo(dT) | oligodeoxythymidylic acid |
| OMT | *O*-methyltransferase |
| ORF | open reading frame |
| PAI | phosphoribosyl anthronilase isomerase |
| PAL | phenylalanine ammonia-lyase |
| PCI | phenol:chloroform:isoamyl |
| PCR | polymerase chain reaction |
| pfu | plaque-forming units |
| poly(A)$^+$ | polyadenylated (mRNA) |
| POX | peroxidase |
| Pti1 | Pto interacting 1 |
| PVPP | polyvinylpolypyrrolidone |
| R gene | resistance gene |
| RACE | rapid amplification of cDNA ends |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| ROS | reactive oxygen species |

| | |
|---|---|
| rpm | revolution per minute |
| RT | reverse transcriptase |
| SAH/SHH | S-adenosyl-L-homocysteine hydrolase |
| SAR | systemic acquired resistance |
| SDS | sodium dodecyl sulfate |
| sec | second |
| SMS/SAM | S-adenosyl-L-methionine synthetase |
| SSC | sodium chloride/sodium citrate |
| TAE | Tris/acetate buffer |
| TEMED | *N,N,N',N'*-tetramethyl-ethylenediamine |
| TMV | tobacco mosaic virus |
| TPNS | triisopropylnaphtalene sulfonic acid |
| Tris-CL | Tris hydrochloride |
| TyrDC | tyrosine decarboxylase |
| UTR | untranslated region |
| µg | microgram |
| µl | microliter |
| UV | Ultraviolet |
| VLCFs | very long chain fatty acids |
| V/v | volume per volume |
| WGA | wheat germ agglutinin |
| w/v | weight per volume |

# CHAPTER I

## INTRODUCTION

Palm oil is one of the 17 major oils and fats that are produced and traded in the world. Malaysia, with 8.315 million tones of palm oil produced in 1998 is the world's leading producer of palm oil, which accounted for 49.5% of the total palm oil produced in the world (Basiron *et al.*, 1999). The oil palm industry in Malaysia is expected to attain a production level of 12.1 million tones by the year 2020 (Basiron *et al.*, 1999).

Various diseases caused by bacterial and fungus that attack oil palm have been reported in Malaysia. Among these, basal stem and root rots caused by *Ganoderma* have resulted severe losses in Malaysia as well as South East Asia. It is important that effective techniques and strategies are developed in order to control plant diseases. However, the application of the molecular biology approach to enhance disease resistance in oil palm is in its infancy. In addition, there is still very limited information on the molecular biology of defense or stress response mechanisms in oil palm that are available. Besides, no studies have reported on defense or stress responsive genes expression in oil palm, especially those related to the defense signal transduction pathway and secondary metabolites biosynthesis.

Understanding the mechanisms of plant defense response and the function of disease resistance genes that govern the resistance of plants to pathogens or stresses have advanced rapidly recently. Major advances in cloning and sequencing of the related genes are providing valuable information on the basis of their roles in defense

mechanisms and recognitional specificities. This further offer the opportunity to engineer genes that regulate an array of defense responses, and enhanced resistance can be obtained with the transformation of cloned defense response genes into crop species to produce transgenic lines.

Isolation and characterization of cDNA clones encoding disease resistance or stress responsive genes is a preliminary step in the commencement of a molecular biological study of oil palm defense response mechanisms. Therefore, fundamental research on genes involved in the defense signal transduction pathway, phenylpropanoid biosynthesis, and genes that encode pathogenesis-related (PR) proteins is needed to provide a better understanding of the molecular mechanisms involved during oil palm defense responses.

The objective of this study was to isolate and characterise defense response genes from an oil palm zygotic embryo cDNA library and suspension culture cDNA library. The isolated genes were characterized for their stress response properties by studying their expression patterns during different stress treatments through northern blot analysis. Besides, the copy number of these genes that are present in the oil palm genome was also estimated.

Therefore, it is hoped that this preliminary study will provide a more meaningful understanding of the genes involved during oil palm defense and stress response mechanisms, thus allow this information to be utilized and be applied to facilitate engineering for disease resistance in oil palm in the future.

# CHAPTER II

# LITERATURE REVIEWS

## The Botany of Oil Palm

The oil palm (*Elaeis guineensis* Jacq.) is grouped under the same tribe with coconut, *Cocoineae*, in the family of *Palmae*. The oil palm originated from the Guinea Coast, West Africa, was first introduced to Malaysia in the year 1871 (Hartley, 1988). The main commercial planting materials in Malaysia were tenera' s (dura x pisifera) since 1961 (Tan, 1983).

The palm has a crown of pinnate fronds on a vascular stem (Corley & Gray, 1976a). There is only one terminal growing point in oil palm (Rees, 1964). The apical meristem is located at the centre of a shallow depression formed by the primordial leaves and other tissues within the crown of the palm. It gives rise to very little stem tissue, producing mainly leaf primordia (William & Hsu, 1970). The leaf primodium arises as a lateral structure from the shoot apex and will give rise to the petiole, rachis and lamina wings or leaflets. The fronds are arranged in two opposing sets of spirals, whereby every $8^{th}$ frond falls on one spiral and every $13^{th}$ on the opposing set (Hartley, 1977). The mature palm may carry a crown of 25-40 fronds. The frond is attached to the stem by a broad clasping base, which narrows into a spiny petiole. The stalk is continued right through the length of the frond as the rachis. Stem tissue is formed at a later stage from a meristem, which is continuous with the bases of the fronds. Oil palm has a typical fibrous root system common to monocotyledonous plants (William & Hsu, 1970).

The palm is monoecious bearing male and female inflorescences in alternating cycles. A hermaphrodite inflorescence may also occur at certain stages. In a potentially female primodium, the two accompanying male flowers are suppressed and remained dimentary. In a potential male primodium, the female organ is suppressed (Beirnaert, 1935). An inflorescence primordium forms in each frond axil about 36 months before it appears externally. As it emerges from the leaf axil, the inflorescences are enclosed in a woody spathe which then splits open to expose the flowers. The female flower consists of a perianth of six segments in two whorls, a tricarpellate ovary and a trifid stigma. The receptive faces of stigma lobes only open out when mature. The male flower also has six perianth parts and six stamens with four locular anthers, which contain masses of pollen. Pollinated flowers take about five to six months to mature. Fertilized flowers produce one fruit that grows and ripens in about six months (Wood, 1986).

The mature pollen grains have two cells, a generative cell and a vegetative cell (Tan, 1976). As the pollen germinates, the generative cell divides giving rise to two male gametes with chromosome complement (n), 16 (Tan, 1976). One of the gametes fertilizes the egg to form a zygote, whereas the other forms the primary endosperm nucleus. The zygote undergoes mitotic divisions to produce an embryo. One of the three ovules in the tricarpellary ovary is fertilized to produce one seed within a fruit, whereas the others degenerate. Oil formation in the endosperm and mesocarp occurs about 70 days after fertilization (Tan, 1983).