



UNIVERSITI PUTRA MALAYSIA

**ISOLATION AND CHARACTERISATION OF THIONIN GENES IN
RICE (ORYZA SATIVA)**

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**ISOLATION AND CHARACTERISATION OF THIONIN GENES IN RICE
(*ORYZA SATIVA*)**

**By
CHIEW JASON**

**Thesis Submitted in Fulfilment of the Requirements for the Degree of
Master of Science in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia**

April 2001



DEDICATIONS

For my family and Li Yen

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: Dr. Suhaimi Napis

Faculty: Food Science and Biotechnology

Thionins are cysteine rich, basic and toxic proteins that are assumed to be involved in the defense against pathogens. Two partial cDNA clones encoding for putative thionin in rice were isolated from endosperm (Rth-1) and shoots (Rth-2). The nucleotide sequences encoding for thionin precursor were further identified from the amplification of the 3'-end of the cDNA. The amino acid sequence for JRth-1 isolated from endosperm showed 72% homology to barley leaf thionins DB4 (Bohlmann and Apel, 1987), 68% to pKG1940 (Gausling, 1987), 51% to hordothionin Hth-1 (Rodriguez-Palenzuela *et al.*, 1988), 50% to purothionin Pur-A1 (Ohtani *et al.*, 1977), 33% to crambin Thi2Ca1 (Teeter *et al.*, 1981) and viscotoxin Vis A3 (Samuelsson *et al.*, 1974).

The homologies for JRth-2 at the amino acid level were 83% to barley leaf thionins DB4 (Bohlmann and Apel, 1987), 71% to pKG1940 (Gausling, 1987), 46% to the hordothionin Hth-1 (Rodriguez-Palenzuela *et al.*, 1988) and purothionin Pur-A1 (Ohtani *et al.*, 1977), 41% to crambin Thi2Ca1 (Teeter *et al.*, 1981) and 33% to the viscotoxin Vis A3 (Samuelsson *et al.*, 1974). Like other thionin precursors, the putative rice thionin has an N-terminal domain characteristic of a signal peptide and



an acidic C-terminal extension. In the expression studies, rice thionins mRNA were found to accumulate abundantly in endosperm and shoots only with a transcript size of around 600-700 bp. In both of these tissues, the expression of the thionin genes was strongly repressed by the exposure to light. Southern analysis showed that the rice thionin seemed to be coded by 1-2 genes.

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

**PENGASINGAN DAN PEMGAJIAN GEN-GEN THIONIN DALAM PADI
(*ORYZA SATIVA*)**

Oleh

CHIEW JASON

April 2001

Pengerusi: Dr. Suhaimi Napis

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Thionins adalah protein yang kaya dengan molikul cysteine, bersifat bes, toksik dan dipercayai terlibat dalam penahanan terhadap serangan patogen. Dua klon separa cDNA yang dianggap mengandungi rangkaian bacaan kepada peptida thionin padi berjaya diasingkan daripada bahagian endosperma (Rth-1) dan daun (Rth-2). Justeru, turutan cDNA yang mengkodkan protein thionin dikenal pasti dengan menggunakan teknik amplifikasi penghujung 3' jalur cDNA. Jujukan untuk klon yang diperolehi daripada bahagian endosperma (JRth-1) mempunyai persamaan dari segi asid amino sebanyak 72% berbanding kepada thionin daun barli DB4 (Bohlmann and Apel, 1987), 68% kepada pKG1940 (Gausling, 1987), 51% kepada hordothionin Hth-1 (Rodriguez-Palenzuela *et al.*, 1988), 50% kepada purothionin Pur-A1 (Ohtani *et al.*, 1977), 33% kepada crambin Thi2Ca1 (Teeter *et al.*, 1981) dan viscotoxin Vis A3 (Samuelsson *et al.*, 1974).

Untuk jujukan cDNA yang dikesan daripada daun (JRth-2), persamaan dari segi turutan protein adalah sebanyak 83% berbanding kepada thionin daun barli DB4 (Bohlmann and Apel, 1987), 71% kepada pKG1940 (Gausling, 1987), 46% kepada hordothionin Hth-1 (Rodriguez-Palenzuela *et al.*, 1988) dan purothionin Pur-A1

(Ohtani *et al.*, 1977), 41% kepada crambin Thi2Ca1 (Teeter *et al.*, 1981) dan 33% to viscotoxin Vis A3 (Samuelsson *et al.*, 1974)

Kedua-dua klon tersebut mempunyai turutan asid amino yang menyerupai protein thionin yang dikenalpasti sebelum ini dengan mempamerkan satu jujukan-N yang bersifat isyarat untuk protein thionin dan satu penghujung-C yang bersifat asidik. Dalam kajian expressi gen thionin dalam padi, isyarat sebahagian besar mRNA dapat dikesan dalam lingkungan saiz 600-700 bp pada endosperma dan daun sahaja. Pemerhatian gen tersebut menunjukkan expressi gen thionin padi dapat direncatkan melalui sinaran cahaya putih. Kajian “Southern Blot” pula menunjukkan gen thionin tersebut berkemungkinan dikodkan oleh satu hingga dua gen sahaja.

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I certify that an Examination Committee met on 27th April 2001 to conduct the final examination of Chiew Jason on his Master of Science thesis entitled "Isolation and Characterisation of Thionin Genes in Rice (*Oryza sativa*)" 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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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.

Chiew Jason

Date: 27 April 2001

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LIST OF ABBREVIATIONS

°C	-	degree centigrade
A	-	adenine base nucleotide
bp	-	base pair
C	-	cytosine base nucleotide
Ca ²⁺	-	calcium ion
cDNA	-	complementary DNA
Ci	-	curie
C-terminal	-	carboxyl terminal
DEPC	-	diethyl pyrocarbonate
DMSO	-	dimethylsulfoxide
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide triphosphate
DTT	-	dithiothreitol
EDTA	-	ethylenediamine tetraacetate
EGTA	-	Ethylene Glycol Bis (2-aminoethylether-N,N,N',N' - Tetraacidic acid)
EtBr	-	ethidium bromide
G	-	guanine base nucleotide
HCl	-	hydrochloride acid
IPTG	-	isopropyl-β-D-thio-galactopyranoside
IRRI	-	International Rice Research Institute
kb	-	kilo base
LB	-	Luria Bertani



LD-PCR	-	long distance polymerase chain reaction
LiCl	-	lithium chloride
M	-	molar
MARDI	-	Malaysia Agricultural Research and Development Institute
MgSO₄	-	magnesium sulphate
MMLV	-	Maloney Murine Leukemia virus
mRNA	-	messenger RNA
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
PEG	-	polyethylene glycol
pfu	-	plaque forming unit
RNA	-	ribonucleic acid
rpm	-	revolution per minute
SDS	-	sodium dodecyl sulphate
S-H	-	sulfur-hydrogen
S-S	-	sulfur-sulfur
T	-	thymine base nucleotide
UV	-	ultraviolet
v/v	-	volume/volume
w/v	-	weight/volume
X-gal	-	5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase

CHAPTER I

INTRODUCTION

It is estimated that pests and diseases reduce the theoretical maximum yield of rice by 50%. More than 60 diseases are well described and numerous fungi were reported to be associated with rice (Lu and Chang, 1980). Blast remains one of the most widely distributed diseases in rice growing areas. Rice seedlings or plants at the tillering stage are often killed (Ou, 1985). Brown spot is another disease reported in all rice growing countries. The fungal disease was reported to be the major factor contributing to the great "Bengal Famine" of 1942 (Padmanaban, 1973). Other major fungal diseases reported are the sheath blight disease, Downy mildew, Stem rot and Sheath spot.

The two major strategies currently employed for controlling the diseases are the use of chemicals and the use of resistant cultivars. Many new rice cultivars and breeding lines developed have multiple resistances to diseases (Ou, 1977). Genes for disease resistance appears to be independent and no close linkage of genes resistant to one disease have been detected as yet. Improved resistance in rice, which provides staple food for most of the world's population would reduce the amount of pesticides in the environment and help increase the world food supply (Ronald, 1997). The genetic engineering of rice is therefore technically feasible.

A number of genes conferring resistance to a variety of pathogens have been cloned. The most prominent being the *Xa21* gene which represent a novel class of plant

disease genes encoding a putative receptor kinase (Song *et al.*, 1995). Twenty-one genes for resistance to *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) have been identified in rice and eight of these genes are clustered among three linkage groups. Another gene, which also confers resistance to *Xoo*, is the *Xa1*. The gene product contains nucleotide binding sites (NBS) and a new type of leucine-rich repeats (LRR), different from the *Xa21*. Other disease resistance genes isolated from rice include the pathogenesis-related (PR) proteins chitinase and glucanase, protein inhibitors, lectins and phytoalexins.

Thionins are cysteine-rich polypeptides of about 5 kDa that are present in cereal leaves (Bohlmann and Apel, 1987; Gausing, 1987) and in a wide range of plant species (Garcia-Olmedo *et al.*, 1989). Known thionin amino acid sequences have been classified into five structural types (Carmona *et al.*, 1993).

The first type was represented by thionins isolated from endosperm of wheat (purothionins) and barley (hordothionins). At least seven sequences are known of this type (Garcia-Olmedo *et al.*, 1992). The second type of thionins is found in the leaves of the parasitic plant *Pyricularia pubera* (Vernon *et al.*, 1985) and in barley (Bohlmann and Apel, 1987; Gausing, 1987). The leaf thionins display a similar variability among their members. It is reported that this group of thionins is encoded by a complex multigene family. The third type includes the viscotoxin isolated from leaves of European mistletoe (*Viscum album*) and phoratoxins (Samuelsson *et al.*, 1974). The viscotoxins are found to be more related to leaf specific thionin even though *V. album* is distantly related to cereals. The fourth type corresponds to the crambins isolated from the Abyssinian cabbage. It is the only known thionins so far

which exhibit no toxicity and has the same sequence length and disulphide bridge arrangement as the second type. The fifth type is divergent from the other four types and had been isolated from the cDNA library of developing kernels (Castagnaro *et al.*, 1992).

All five types of thionins appear to be highly homologous at the amino acid level. This highly conserved sequence includes the cysteine residues and the tyrosine residues at position 13 (Florack and Steikema, 1994). Except for crambins, the first and the last two amino acid residues in thionins were conserved as well (Bohlmann and Apel, 1991). The possible involvement of thionins in plant defense was first suggested, on the basis of their *in vitro* toxicity to plant pathogens, by Fernandez de Caleyra *et al.*, in 1972. The toxicity of thionins was believed to be based upon their amphipathic structure, which makes them act like detergents (Reimann-Philipp *et al.*, 1989). Besides, the exact biological function of thionins in plants has not been reported so far.

Objectives

The objectives of my project are as follows:

- 1) To isolate and characterise thionin genes from etiolated rice seedlings
- 2) To study the effects of light towards the regulation of the gene

CHAPTER II

LITERATURE REVIEW

Rice

Introduction

Rice is generally considered a semiaquatic annual plant, although it could survive as a perennial in the tropics and subtropics. Cultivars of the 2 cultivated species, *Oryza sativa* L. and *O. glaberrima* Steud., can grow easily in a wide range of conditions (Lu and Chang, 1980). According to figures released by the International Rice Research Institute (2000), global production of rice hovered between a high 484.9 million tons of rough rice in 1988 and 534.7 million tons in 1994. The rate of increase in production during the 80's (2.8%) was slightly lower than that of the previous decade (3.1%) (Lu and Chang, 1980). The decline during 1987 was mainly due to adverse weather conditions in many rice growing countries. The peak in world rice production of about 534.7 million tons in 1994 was largely achieved by Asian countries (90.72%). Malaysia alone contributed 2 million tons to the global production (IRRI, 2000). The cultivars were mainly the semidwarf varieties and hybrid rice (grown in China) which filled more than 70 million ha of Asian riceland (Lu and Chang, 1980).

The Origin and Evolution of Rice

The genus *Oryza* includes 20 valid species. Various synonymous of the *Oryza* species under several classification and nomenclature systems proposed by different botanist were listed by Chang (1964), Nayar (1973) and Oka (1975). A biosystematic treatment of species in the genus has been made by Sampath (1962), Chang (1964), Oka (1974) and Nayar (1973). Morphologically, *O. glaberrima* differs from *O. sativa* in having shorter ligules, absence of secondary branching on the panicles, glabrous glumes and slightly scabrid leaves, although the geographic distribution of the 2 cultivated species appears distinct (Lu and Chang, 1980).

Rice Plant Growth and Development

The life cycle of the rice plant is generally from 100 to 210 days, depending on the variety and the environment under which it is grown. In temperate climates, the average duration from sowing to harvest is about 130 to 150 days (Vengara, 1991). Cultivars with duration of 150 to 210 days are usually photoperiod sensitive and planted in the deepwater areas. Temperature and day length are the two environmental factors affecting the development of the rice plant.

According to Vengera (1970), development of rice plant can be divided into 3 main phases; vegetative phase (from seed germination to panicle initiation), reproductive phase (from panicle initiation to anthesis) and ripening phase (from anthesis to full maturity). The vegetative phase is characterized by active tillering, gradual increase in height and leaf emergence at regular intervals. The reproductive growth phase is

characterized by culm elongation, decline in tiller number, emergence of leaves and flowering of the Spikelets. Spikelets anthesis or flowering begins with panicle exertion that usually takes 7-10 days to complete (IRRI, 2000).

Rice Diseases

Introduction

Rice diseases are major biological constraint in rice production. More than 60 diseases are well described and numerous species of fungi are reported to be associated with rice. They are caused by various pathogens such as viruses, bacteria, fungi, nematodes and others (Ou, 1972). Some diseases are cosmopolitan while others are confined to certain regions. Crop losses from diseases can be severe.

Blast is one of the most widely distributed diseases in rice growing areas. Rice seedlings or plants at the tillering stage are often completely killed. Heavy infections on the panicles are often detrimental to the rice yields. The causal organism, *Pyricularia oryzae*, was first named by Cavara in Italy in 1981 (Ou, 1985). Briosi and Cavara describe the conidia as sporiferous hyphae usually scattered and round in shape. The size of the conidia varies considerably among isolates and from different environmental conditions. The fungus produces spots or lesions on leaves, nodes and different parts of the panicles and the grains. The predominant symptoms of blast disease depend upon the climatic conditions. In the temperate regions, leaf blast at the tillering stage often kills the plants. In the tropics, severe infections are seldom found after transplanting the seedlings in nurseries (Ou, 1985).

Brown spot is another disease reported in all rice growing countries in Asia, America and Africa (Ou, 1972). The fungal disease causes blight of seedlings grown from heavily infected seeds. The disease was reported to be the major factor contributing to the great “Bengal Famine” of 1942, causing 50-90% losses to the rice growing areas (Padmanaban, 1973). The casual organism of this particular disease is *Cochliobolus miyabeanus*, which is a black velvety mycelial mats distributed irregularly and sparsely through the panicles.

The sheath blight disease has become increasingly important in recent years in most rice growing areas because of the use of high yielding cultivars that produced large numbers of tillars. This resulted in an increase in the humidity of the plant layer and therefore facilitates the infections of this disease (Ou, 1985). The sheath blight disease is caused by *Rhizotonia solani*, a colourless mycelium of varying shapes and sizes. The disease causes spots on the leaf sheath whereby the size and the formation of the spots depend upon the environmental conditions.

Other major fungal diseases reported in the rice growing areas were the Downy mildew (*Sclerophthora macrospora*), Stem rot (*Magnaporthe salvinii*) and Sheath spot (*Rhizotonia oryzae*).

Disease Control in Rice

Two major strategies currently employed for controlling diseases are the use of chemical protection and breeding for resistant rice cultivars (Ou, 1972). In Asia, rice

production has entered an era of change. The use of chemicals for pest management has also increased at an unprecedented rate (Mew, 1988).

In tropical Asia, these chemicals are primarily insecticides and herbicides, while in temperate zone in eastern Asia, disease control represent the biggest share of chemical use (Mew, 1988). However, pathogens have developed resistant strains to chemicals, particularly the blast fungus. This is due to the excessive use of chemicals to counter this disease (Sakura and Naito, 1976).

A more suitable strategy of controlling plant diseases is the use of resistant cultivars. This is particularly appropriate for the small rice farmers in developing nations. Since 1962, the development of disease resistant rice cultivars has received greater attention in international food research programs (Ou, 1972).

In tropical Asia where several diseases occur together, rice cultivars must have resistance to major diseases and to insect pests. At present, many new rice cultivars and breeding lines have multiple resistances to diseases (Ou, 1977). Genes for disease resistance appear to be independent and no close linkage of genes resistant to any of the rice disease have been detected as yet.

The inherent level of resistance alone is too low to control diseases that occur in rice. A cultivar with a moderate level of resistance in combination with proper chemical applications is effective in managing various diseases (Chen *et al.*, 1987). Besides, many antagonistic bacteria were found in the paddy field that effectively suppressed the development of different diseases.

Systemic Acquired Resistance

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens (Ryals *et al.*, 1996). After the formation of a necrotic lesion, either as a part of hypersensitive response (HR) or as a symptom of disease, the SAR is activated. SAR activation results in the development of a broad spectrum of systemic resistance.

In tobacco, SAR activation results in a significant reduction of disease symptoms caused by the fungi *Phytophthora parasitica*, *Cercospora nicotianae*, *Peronospora tabacina*, the viruses tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), and the bacteria *Pseudomonas syringae* and *Erwinia carotovora* (Vernooij *et al.*, 1995). However the protection is not effective against all pathogens. Ryals (1996) reported that there is no significant protection of SAR against either *Botrytis cinerea* or *Alternaria alternata*. According to Ward (1991), the expression of a set of genes called SAR genes are associated with SAR. However not all defense related genes are expressed during SAR. The particular spectrum of gene expression therefore distinguishes the SAR response from other resistance response in plants.

The SAR signal transduction pathway appears to function as a potentiator or modulator of other defense mechanisms. When SAR is activated, a normally compatible plant-microbe interaction can be converted into an incompatible one (Uknes *et al.*, 1992; Mauch-Mani and Slusarenko, 1996). The mechanism by which this modulation occurs remained unknown.

