UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF A GENE CODING FOR CHITINASE IN DEVELOPING WINGED BEAN SEED (PSOPHOCARPUS TETRAGONOLUBUS)

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By

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Chairman: **Suhaimi Napis, Ph.D**

Faculty: **Food Science and Biotechnology**

Chitinase, which catalyse the hydrolysis of the β-1,4-N-acetyl-D-glucosamine linkages of the fungal cell wall polymer chitin, is involved in inducible defences of plants. The aim of this research is to isolate a chitinase genes from seed of winged bean. In order to isolate genes encoding for chitinases from a cDNA library of winged bean seed, two sets of degenerate primers were designed which corresponded to conserved regions of chitinases class I and class IV. These were then reverse transcribed and subsequently amplified using polymerase chain reactions (RT-PCR) using 4 weeks old seeds cDNA as template. A 1.1 kb fragment was cloned, and subjected to terminal sequence analysis to verify the presence of sequences encoding for chitinases. Nucleotide sequence analysis

showed that the fragment appears to encode for a class I chitinase, and this fragment was then used as a probe to screen for a full length gene from the winged bean seed cDNA library.

After library screening one clone, CHRZP was isolated and found to encode a chitinase gene. The complete nucleotide sequence of the winged bean chitinase (1324 bp) encoded a polypeptide of 289 amino acids that encodes for a basic chitinase with cysteine-rich domain at the N-terminal. A Comparison of amino acid sequence showed 88% similarity to a chitinase sequence isolated from *Oryza sativa*. RNA blot hybridisation revealed that mRNA that corresponded to CHRZP accumulates to high levels in leaves compared to seed, tuber and pod with a transcript size of 1.0 kb. Southern hybridisation analysis indicated that this gene was present as a single copy gene in the winged bean genome.

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

PEMENCILAN DAN PENCIRIAN GEN CHITINASE SEMASA PEMBENTUKAN BIJI KACANG BOTOL (*PSOPHOCARPUS TETRAGONOLOBUS*)

Oleh

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Chitinase adalah enzyme yang menghidrolisis ikatan β-1,4-N-acetyl-D-glucosamine polymer chitin yang terdapat pada dinding sel fungi yang mana terlibat dalam sistem pertahanan tumbuhan. Tujuan utama kajian ini dilakukan ialah untuk memencilkan gen chitinase daripada biji kacang botol. Untuk memencilkan jujukan yang mengkod chitinase dalam perpustakaan cDNA daripada biji kacang botol, 2 set primer yang terubahsuai (degenerate) telah direka sepadan dengan rantau chitinase kelas I dan kelas IV. Ianya digunakan di dalam tindakbalas pempolimeran rantai secara pembalikan transkripsi (RT-PCR) dengan menggunakan cDNA daripada biji yang berusia 4 minggu sebagai template. Fragmen yang dihasilkan yang bersaiz 1.1 kb telah diklonkan dan dianalisis untuk

mengenalpasti kehadiran jujukan yang mengkod gen chitinase. Jujukan nukleotida yang dianalisis daripada fragmen tadi menunjukkan ianya mengkodkan gen chitinase kelas I dan fragmen ini seterusnya digunakan sebagai probe untuk pencirian perpustakaan cDNA daripada biji kacang botol.

Selepas pencirian satu klon, CHRZP yang mengkod untuk gen chitinase berjaya dipencilkan. Jujukan nukleotida yang lengkap untuk gen chitinase daripada kacang botol (1328) mengandungi 289 asid amino dan ianya mengkodkan chitinase beralkali dengan domain yang kaya dengan sistein pada kedudukan N-terminal. Perbandingan jujukan asid amino menunjukkan ianya mempunyai 88% persamaan dengan jujukan chitinase daripada *Oryza sativa*. Penghibridan RNA menunjukkkan bahawa klon CHRZP mempunyai ekspresi yang tinggi dalam daun berbanding dengan biji, kulit, dan ubi dengan saiz transkripsi 1.0 kb. Analisis penghibridan Southern menunjukkan kehadiran satu salinan gen ini di dalam genom kacang botol.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
A_{230}	Absorption at 230 nm
A_{260}	Absorption at 260 nm
A_{280}	Absorption at 280 nm
bp	Base pair
cDNA	Complementary DNA
CHRZF	Forward primer for RTPCR
CHRZR	Reverse primer for RTPCR
CHRZP	Chitinase clone
CTAB	Cetyl trimethyl ammonium bromide
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
IPTG	Isoprophylthiogalactoside
kb	Kilobase pair
LacZ	β-galactosidase gene
MCS	Multi cloning site
MOPS	3(N-morpholine) propane sulfonic acid
mRNA	Messenger RNA
NaCl	Sodium chloride

OD	Optical density
Oligo(dT)	Oligodeoxythymidylate
PCR	Polymerase chain reaction
Phage	Bacteriophage
PVP	Polyvinylpyrrolidone
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TE	Tris-EDTA buffer
tRNA	Transfer RNA
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyronoside
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Fungal pathogens cause heavy crops losses amounting to several billion dollars. Molecular biology of pathogenesis is an important field that has changed the disease management strategy from chemical control to development of transgenic disease-resistant plants or "induced systemic resistant" plant. Several genes from either plants or microorganism (bacteria and fungi) encoding proteins with *in vitro* antifungal activity have been analysed. However only in a few cases it could be demonstrated that the observed *in vitro* antifungal activity correlated with *in vivo* protection in transgenic plants (Cerny, 1980).

The defense responses of plant during infection with fungal pathogen include the inducible synthesis of a number of proteins which, directly or indirectly, may participate in the active protection against the invading pathogen. Among these proteins are the hydrolytic enzymes, e.g. chitinase and β-1,3-glucanase. It has been well established that plant chitinases and β-1,3-glucanase have the potential to partially degrade fungal cell walls. The products formed are oligosaccharides, and it is possible that such oligosaccharides are perceived by the plant cell as a signal, so-called elicitors to induce active defense responses.

The primary aim of this project was to isolate a chitinase gene from winged bean, which encode a protein that is involved in providing resistance to fungal diseases. Chitinases hydrolyze the beta-1,4 linkages of chitin, a biopolymer of N-acetyl -D-glucosamine. Chitin is a cell wall component of many phytopathogenic fungal species. Because plants lack endogenous chitin, plant chitinases are thought to play an antifungal role. This defensive role for plant chitinases has been supported by *in vitro* studies (Benhamou *et al.*, 1993), *in vivo* studies (Rasmussen *et al.*, 1992) and transgenic experiments (Broglie *et al.*, 1991).

Many chitinases have been characterized from agriculturally important crops including tobacco (Shinshi *et al.*, 1990), bean (Broglie *et al.*, 1986), rice (Nishizawa and Hibi, 1991) and corn (Huynh *et al.*, 1992). We are interested in identifying and characterizing the natural defense systems of local winged bean (*Psophocarpus tetragonolobus*), and have decided to focus on the role of chitinases in the protection of local winged bean from pathogens. Winged bean was choosen in this study because winged bean appears to have great potential for easing the problem of protein malnutrition. By comparing the composition and nutritional value of soybeans and winged bean; both contain the similar proportion of protein, oil, minerals, vitamins, essential amino acids, and other constituents. The winged bean may one day become as significant as the soybean in world agriculture. Compared to other parts of winged bean, the winged bean seed has created the greatest interest internationally, because of its high content of protein and oil.

The specific objectives of this study were:

(1) To develop a suitable protocol for the isolation of total RNA from winged bean tissue.

(2) To construct a cDNA library from seed of winged bean.

(3) To identify and isolate a chitinase gene from the cDNA library.

(4) To study the expression of the chitinase gene in winged bean tissue.

(5) To determine the copy number of this gene in the genome of winged bean.

CHAPTER II

LITERATURE REVIEW

Winged Bean: A Potential Crop

Winged bean, *Psophocarpus tetragonolobus (L) D.C.* is a member of the Leguminosae family and grows well in tropical countries such as Papua New Guinea and Southeast Asia. It grows abundantly in hot, humid equatorial countries such as Indonesia, Malaysia, Thailand, Philippines, India, Bangladesh, Burma and Sri Lanka. Although at one time it is considered as a "poor man's food", the potential economic importance of the plant has attracted worldwide attention and is now recognized as a "high protein crop for the tropics" (Cerny, 1980).

Winged bean plant is a climber with cines and leaves, which is 3-4 m in length. It is a herbaceous perennial; but can be grown as an annual plant. There are several cultivars with wide differences in physical features and in physiology. The plant produces an abundance of leaves and inflorescences of white, blue, deep purple or pink flowers, which quickly develop into pods. The pods are 4-sided with fringed wings and can be 6-30 cm in length and carrying 5-20 seeds per pod. The seeds, which are rich in protein, are comparable to soybean in composition and nutritional value and contain similar proportions of protein (30-40%), carbohydrates, oil (15-20%), minerals, vitamins, essential amino acids and others.

Besides the various economical and industrial uses of seeds for commercial exploitation, they are also useful as food when steamed, boiled, fried, roasted, fermented, made into milk or prepared by other methods. The plant also produces underground tubers of varying sizes and richs in carbohydrates (25-30%) and proteins (10-15%). The plant is one of the best nitrogen fixers with nodulation accomplished by soil bacterium, *Rhizobium* and a because of its ability to fix nitrogen from the atmosphere, the plant requires very little or no fertilizers (Claydon, 1978).

Callus tissue and suspension cultures of the winged bean have been established in a salt-sucrose culture medium supplemented with various combinations of auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) + kinetin and/or 6-benzylaminopurine (BA). Regeneration of plantlets was been achieved both via organogenesis and somatic embryogenesis. Little research has been carried out on the winged bean and as such information is limited. Winged bean appears to have great potential for solving the problem of protein malnutrition throughout the humid tropics. Compared to other organs of winged bean, the winged seeds have created the greatest interest internationally. They virtually duplicate soybeans in composition and nutritional values; both contain similar proportions of protein, oil, minerals, vitamins, essential amino acids, and other constituents. The winged bean may one day become as significant as the soybean in world agriculture (Cerny, 1980).

Taxonomy

Psophocarpus is a genus with about nine species (Cerny, 1980). Only *P. tetragonolobus* and *P. palustris* have been used for food. The other species have never been cultivated. Even *P. palustris* remains a semiwild plant, used in West Africa mainly in times of famine.

Biological Variation

Recent collections of winged beans from different parts of Asia revealed wide differences in physical features such as; leaf shape and size, flowers colour, pod length, shape, and colour; wing shape and surface texture; seed shape, size and colour; tuber size; and stem colour. There are also physiological differences: time required for seeds to germinate, flowers to form, pods to set, seeds to mature, and tuber to form. In addition, there are variations in the protein, oil, and other components of the seeds and other parts of the plant (Stephenson *et al.*, 1979).

Food Use and Nutritive Value

The amounts of major nutrients, such as protein, minerals, and vitamins, in the various winged bean parts are shown in Table 1. The common characteristic of all parts of the winged bean is the relatively high protein content. Seeds and are particularly rich in protein.

Table 1: Macromolecules Composition in Different Parts of the Winged Bean

	Flowers	Leaves	Immature Pods	Unripe Seeds	Ripe Seeds	Tubers
Water[a]	84.2-87.5	64.2-85.0	76.0-93.0	35.8-88.1	8.7-24.6	54.9-65.2
Energy[b]	0.17	0.20	0.19	0.10-0.71	1.61-1.89	0.63
Protein[b]	2.8-5.6	5.0-7.6	1.9-4.3	4.6-10.7	29.8-39.0	3.0-15.0
Fat[b]	0.5-0.9	0.5-2.5	0.1-3.4	0.7-10.4	15.0-20.4	0.4-1.1
Carbor-hydrat[b]	3.0-8.4	3.0-8.5	1.1-7.9	5.6-42.1	23.9-42.0	27.2-30.5
Fiber[b]		3.0-4.2	0.9-3.1	1.0-2.5	3.7-16.1	1.6-17.0
Ash[b]	0.8	1.0-2.9	0.4-1.9	1.0	3.3-4.9	0.9-1.7

Source: Cerny, 1980
Note: [a] Values expressed as g per 100 g fresh weight
[b] mj=megajoules. 4.184 mj=1,000 (dietary) kilocalories

The immature pod provides the bulk of comparatively low energy content, but is beneficial as a vegetable because of the mineral and vitamins it contains (Cerny and Addy, 1973).

Seeds

The mature dry seeds are the most nutritious part of the winged bean. Their outstanding nutritive quality is based, above all, on their high protein content (34-42%) and amino acid composition. The seed also contains a large amount of edible oil (15-20%). With the exception of the soybean and the peanut, no other commonly consumed food legume can rival the winged bean in the combination of protein and oil.