UNIVERSITI PUTRA MALAYSIA

MOLECULAR CLONING, FUNCTIONAL ASSESSMENT AND TRANSCRIPTOME CHARACTERIZATION OF CYSTEINE PROTEASE INHIBITOR AND KUNITZ TRYPSIN INHIBITOR FROM TURMERIC (Curcuma longa L.)

CHAN SEOW NENG

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By

CHAN SEOW NENG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2016

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MOLECULAR CLONING, FUNCTIONAL ASSESSMENT AND TRANSCRIPTOME CHARACTERIZATION OF CYSTEINE PROTEASE INHIBITOR AND KUNITZ TRYPSIN INHIBITOR FROM TURMERIC (*Curcuma longa* L.)

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Protease inhibitors (PIs) are biochemical compounds commonly found in all organisms; function to regulate proteases activities in cell. Plant PIs such as cysteine protease inhibitor (CYP) and Kunitz trypsin inhibitor (KTI) have been reported to show anti-pathogenic properties and are responsible for the plant tolerance against various stresses. Similar to other plant defence-related proteins, numerous plant PIs genes have been characterized and expressed as recombinant proteins for industrial applications or genetically inserted into crop plants for phytoprotection. However, the effectiveness of these applied plant defence-related proteins (including PIs) in combating the targeted pathogens slowly weakens as there are continuous adaptation processes and resistance developed by pathogens. One important approach to overcome this is to discover and incorporate the use of new PI genes from novel sources where they have not been exposed to the pathogens before. The objectives of this study are to characterize novel PI genes from turmeric, *Curcuma longa*, as turmeric extracts were reported to contain numerous medicinal properties including inhibition of viral proteases. The identified PI genes were to be expressed as recombinant proteins and their functional activities were assessed. Besides, this study also aimed to generate a transcriptomic library of *Curcuma longa* for future molecular biology works. Initially, complementary-DNA (cDNA) fragments of CYP and KTI were identified from leaf samples of *Curcuma longa* treated with methyl jasmonate by using degenerate polymerase chain reaction (PCR). The full-length cDNAs, designated *CypCl* and *ClKTI*, were obtained by using rapid amplification of cDNA ends method (RACE) with complete open reading frames (ORFs) detected. The cDNA sequences have been deposited in NCBI database with the accession number KF545954.1 (*CypCl*) and KF889322.1 (*ClKTI*). The expression profile of *CypCl* and *ClKTI* genes in methyl jasmonate treated tissues was determined by using Real-Time quantitative-PCR (RT-qPCR) and it was found that the PIs genes were generally induced in all treated tissues. Both PIs were cloned into expression vectors and recombinant protein expressions were optimised in the bacterial-host systems. Optimum incubation temperature and concentrations of isopropyl β-D-1-

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thiogalactopyranoside (IPTG) used for the recombinant protein expression was determined at 37ºC and 0.5 mM of IPTG for 2 hours induction. The recombinant *CypCl* was expressed as soluble protein while recombinant *ClKTI* was expressed as inclusion bodies. Recombinant *CypCl* was purified using immobilized metal affinity chromatography (IMAC) and anion-exchange chromatography while recombinant *ClKTI* was solubilized and refolded before purification by IMAC. The concentration of the purified recombinant *CypCl* obtained was 5 µg/µL and the refolded *ClKTI* was 1 µg/µL. The purified recombinant proteins were shown to possessed protease inhibitory activities but did not show obvious anti-pathogenic potential from screenings performed using agar plate well-diffusion method. Lastly, transcriptomic library of *Curcuma longa* was generated through next-generation sequencing (NGS), RNA-seq, from RNA of differently treated leaf samples using Illumina Hiseq platform. The *de novo* transcriptome assembly was conducted from the combined raw RNA-seq reads by using Trinity software and resulted in 113,209 assembled transcripts. Around 50% of the assembled transcripts were found to be functional annotated which included gene ontology (GO) terms annotation by the sequence homology search performed on known proteins from different databases by using Trinotate software. The differential expressed genes (DEG) in the methyl jasmonate-treated sample as compared to the control were identified with 1,559 upregulated transcripts and 2,715 downregulated transcripts. In conclusion, two novel PI genes (CYP and KTI) were identified from turmeric and their expressions profiling were characterized. Recombinant expression in *E. coli* system, refolding and purification of PIs were optimised and inhibitory activities were suggested from the inhibitory assays while weak to resistance anti-pathogenic potential were concluded from the tested fungi. Finally, a transcriptome library of turmeric was obtained and the transcripts were annotated and analysed.

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PENGKLONAN MOLEKULAR, PENILAIAN FUNGSI DAN PENCIRIAN TRANSKRIPTOMIK PERENCAT PROTEASE SISTEINA DAN PERENCAT TRIPSIN KUNITZ DARI KUNYIT (*Curcuma longa* L.)

Oleh

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Perencat protease (PI) merupakan sebatian biokimia yang biasa dijumpai dalam semua organisma yang terlibat di dalam pengawalaturan aktiviti protease sel. PI tumbuhan seperti perencat protease sisteina (CYP) dan perencat protease Kunitz (KTI) telah dikenalpasti mempunyai ciri anti-patogenik dan bertanggungjawab dalam toleransi terhadap pelbagai jenis tekanan pada tumbuhan. Seperti protein berkait pertahanan yang lain, kebanyakkan PI tumbuhan telah diciri dan diekpreskan sebagai protein rekombinan sebagai aplikasi industri ataupun kejuteraan genetik untuk fitopertahanan. Namun begitu, keberkesanan protein ini (termasuk PI) dalam melawan patogen menjadi lemah secara perlahan-lahan disebabkan wujudnya proses adaptasi berterusan dan rintangan yang terbina oleh patogen. Satu pendekatan penting bagi mengatasi masalahan ini adalah dengan mencari dan menggabungkan penggunaan gen PI baru dari sumber novel dimana ianya masih belum terdedah kepada patogen. Objektif kajian ini adalah untuk mencirikan gen-gen PI novel dalam kunyit, *Curcuma longa*, kerana ekstrak kunyit telah dilaporkan mengandungi pelbagai sifat perubatan termasuk perencatan protease viral. Seterusnya, gen PI yang ditemui itu diekspreskan sebagai protein rekombinan dan fungsi aktivitinya dikenalpasti. Kajian ini juga bertujuan untuk menghasilkan perpustakaan transkriptomik *Curcuma longa* bagi kerja-kerja biologi molekul pada masa hadapan. Pada dasarnya, jujukan serpihan komplementari-DNA (cDNA) bagi CYP dan KTI telah dikenalpasti dari sampel daun *Curcuma longa* yang dirawat dengan metil jasmonat melalui tindakbalas polimerase berantai (PCR) degenerat. Jujukan penuh rangka bacaan terbuka (ORFs) cDNA bagi *CypCl* dan *ClKTI*, telah diperolehi melalui kaedah *rapid amplification of cDNA ends* (RACE). Jujukan cDNA tersebut telah didepositkan dalam pangkalan data NCBI dengan nombor aksesi KF545951.1 (*CypCl*) dan KF889322.1 (*ClKTI*). Profil pengekspresan gen-gen *CypCl* dan *ClKTI* dalam tisu yang telah dirawat dengan metil jasmonat ditentukan melalui kuantitatif-PCR masa nyata (RT-qPCR) dan secara umumnya, pengekspran PI sasaran diaruh dalam semua tisu yang dirawat. Kedua-dua ORF PI tersebut diklonkan ke dalam vektor pengekspresan sistem bakteria. Suhu inkubasi dan kepekatan optimum isopropil β-D-1-tiogalaktopiranosida (IPTG) dalam pengekspresan protein rekombinan telah dilakukan pada 37°C dan 0.5 mM IPTG untuk aruhan selama 2 jam. *CypCl* rekombinan

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terekspres sebagai protein larut manakala *ClKTI* rekombinan telah diekspreskan sebagai protein tidak larut (*inclusion bodies*). *CypCl* rekombinan telah ditulenkan menggunakan *immobilized metal affinity chromatography* (IMAC) dan *anion-exchange chromatography* manakala *ClKTI* rekombinan telah disolubilisasikan dan melalui proses perlipatan semula sebelum ditulenkan menggunakan IMAC. Kepekatan *CypCl* rekombinan yang ditulenkan adalah sekitar 5 µg/µL manakala *ClKTI* adalah sekitar 1 µg/µL. Protein rekombinan yang telah ditulenkan tersebut telah menunjukkan aktiviti perencatan protease tetapi tidak menunjukkan potensi anti-patogen yang nyata berdasarkan kaedah pernyaringan yang telah dilakukan iaitu kaedah *well-diffusion* dalam kultur agar. Akhir sekali, perpustakaan transkriptomik *Curcuma longa* telah dihasilkan melalui kaedah *next generation sequencing* (NGS), melalui platform Illumina Hiseq RNA-seq, dengan menggunakan RNA keseluruhan dari sampel daun yang dirawat secara berbeza. Proses himpunan transkriptom secara *de novo* telah dijalankan dengan mengumpulkan kesemua bacaan RNA-seq menggunakan perisian Trinity dan sebanyak 113,209 himpunan transkrip dihasilkan. Fungsi annotasi termasuk annotasi terma ontologi gen (GO) untuk kira-kira 50% daripada himpunan transkrip tersebut telah diketahui melalui pencarian jujukan homolog terhadap protein yang diketahui daripada pangkalan data yang berbeza dengam menggunakan perisian Trinotate. Pembezaan pengekspresan gen (DEG) dalam sampel yang dirawat dengan metil-jasmonat berbanding dengan sampel terkawal telah ditentukan melalui perisian edgeR dengan sebanyak 1,559 transkrip pengawalaturan naik dan sebanyak 2,715 transkrip pengawalaturan turun. Kesimpulannya, dua gen novel PI (CYP dan KTI) telah dikenalpasti dari sample kunyit and profil pengekspresannya telah dicirikan. Pengekpresan rekombinan, perlipatan and penulenan protein PI telah dioptimumkan dan aktiviti perencatan dapat dikesan dari asai perencatan walaupun hanya potensi anti-patogen yang lemah ke rintangan didapati daripada kulat yang diuji. Perpustakaan transkriptomik kunyit telah diperolehi dan transkrip tersebut telah diannotasikan.

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I certify that a Thesis Examination Committee has met on 29 December 2016 to conduct the final examination of Chan Seow Neng on his thesis entitled "Molecular Cloning, Functional Assessment and Transcriptome Characterization of Cysteine Protease Inhibitor and Kunitz Trypsin Inhibitor from Turmeric (*Curcuma longa* L.)" in accordance with the Universities 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 Doctor of Philosophy.

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LIST OF ABBREVIATIONS

3D	Three-dimensional
ABA	Abscisic acid
BAEE	Na-benzoyl-L-arginine ethyl ester
BANA	N-benzoyl-Larginine-2-napthylamide
BBI	Bowman Birk Inhibitor
BLAST	Basic Local Alignment Search Tools
BSA	Bovine Serum Albumin
Bt	*Bacillus thuringiensis*
cAMP	Cyclic adenosinemonophosphate
cDNA	Complementary-deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
CYP	Cysteine protease inhibitor
DEG	Differential expressed gene
dH_2O	Distilled H_2O
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
et al.	Et alia
EtBr	Ethidium bromide
FC	Fold-change
FDR	False discovery rate
GO	Gene ontology
GSP	Gene specific primer
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IDA	Iminodiacetic acid
IEX	Ion exchange chromatography
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl thiogalactopyranoside
IUPAC	International Union of Pure and Applied Chemistry
K_i	Inhibition constant

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K_m	Michaelis constant
KTI	Kunitz trypsin inhibitor
LB	Luria-bertunia
LiCl	Lithium chloride
MARDI	Malaysian Agriculture Research and Development Institute
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
MSI	Mustard trypsin inhibitor
NaCl	Sodium Chloride
NBT/BCIP	Nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
Ni-NTA	Nickel-nitrilotriacetic acid
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
p-DMCA	p-dimethylaminocinnamaldehyde
PI	Protease inhibitor
PVP	Polyvinylpyrrolidon
RACE	Rapid Amplification of cDNA End
RIN	RNA integrity number
RNA	Ribonucleic acid
RT-qPCR	Real-Time quantitative PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ta	Annealing temperature
TAE	Tris-acetate-EDTA
TBST	Tris-Buffered Saline-Tween
TEMED	Tetramethylethylenediamine
UPM	Universiti Putra Malaysia
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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LIST OF NOTATIONS

%	percent
kDa	kilo Dalton
L	litre
α	alpha
β	beta
mL	mililitre
mg	miligram
M	molar
w/v	weight per volume
v/v	volume per volume
µL	microlitre
mg	miligram
g	gram
mM	milimolar
V	volt
bp	base pair
ng	nanogram
OD	optical density
pI	isoelectric point
N50	median of lengths in NGS genome assembly

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CHAPTER 1

INTRODUCTION

Agriculture is one of the important income sources to Malaysia and most of the crop plantings are conducted in a gigantic scale especially for crops like palm oil, rice and rubber trees. It is not uncommon to find pest problems in the plantations and during crop post-harvesting. These amounted to a major loss of profits to the country. Instead of using chemical pesticides which could be harmful to the environment and consumers, the production of crop plants with enhanced traits through biotechnology technique is one of the alternatives to overcome these problems. Advancement in technology had enabled the production of genetically engineered crop plants where the crops acquired beneficial defensive genes from other plants which helped in the protection against pests. One example of these beneficial genes which are responsible in natural plant protection is the gene coding for protease inhibitors.

Protease inhibitors (PIs) are generally defined as molecules that inhibit the proteolysis of protease. Proteolysis is the degradation actions by proteases where large proteins are breakdown into smaller proteins and amino acids. In non-host plant pathogenesis, PIs are released as one of the resistance response by plants when they are attacked by pest insects and microorganisms (van Wyk *et al*., 2016; Benchabane *et al*., 2010; Habib & Fazili, 2007; Heath, 2000). During the process of pathogenesis, pest insects and microorganisms attack the plants by causing mechanical injury and release digestive proteases into the cells of the targeted plant. These released proteases degrade the plant proteins into peptides and amino acids which then formed a large reservoir of nutrients for the pest insects and microorganisms (Schaible & Kaufmann, 2005). The production of PIs are often stimulated by wounding and the PIs produced can be found locally and subsequently other parts distal from the injury area (Koiwa *et al*., 1997).

In recent development, various PIs from plants have been extracted and characterized. Previous studies showed that genes coding for PIs can be identified from the plants genome and some are reported to be utilised in the production of genetically engineered plant that expressed phytoprotection towards certain pest insects or microorganism naturally (Schlüter *et al*., 2010; Christou *et al*., 2006; Dunaevsky *et al*., 2005; Lawrence & Koundal, 2002). In addition, PIs or the recombinant protein of PIs could also be utilised as active compound in biopesticides because they showed anti-pathogenic properties in direct test assays and when ingested by pest insects, a profound reduction in body size was observed (Cruz *et al*., 2013; Rodrigues Macedo *et al*., 2010; Haq *et al*., 2004). Through these applications, the use of chemical pesticides will be reduced and these posed as an environmental friendly alternative apart from a costs saving strategy. Moreover, due to the nature of being able to inhibit proteases, PIs have pharmaceutical potentials and are used as a synthetic product in synthesizing medications especially towards diseases that are implicated by the loss of proteolytic activities of proteases in human body and infection by pathogenic proteases (Scott & Taggart, 2010; Haq *et al*., 2004).

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Nonetheless, over the course of time, pest insect and pathogens are able to develop resistance towards the currently available engineered protection. These applied phytoprotection strategies become less effective in combating the pathogens and could leads to serious damages if precautionary steps are not taken (van Wyk *et al*., 2016; Haq *et al*., 2004). Hence, one of the necessary preparations in overcoming the resistance adaptation of insects and pathogens is to discover more novel PIs genes from novel sources. Novel sources here are referred to plants or organisms which are unrelated to the target crop plants that needed protection (Haq *et al*., 2004). Owing to the PIs novelty, the specific pathogens have not been exposed to them before and therefore the novel PI gained an advantage in combating against the resistance portrayed (Schlüter *et al*., 2010; Christou *et al*., 2006; Haq *et al*., 2004). One of the suitable candidates that could serve as a source for the discovery of novel PIs is the turmeric plant.

Turmeric (*Curcuma longa*) is a well-known traditional medicine and is commonly used as spices in culinary arts in country like India, Malaysia, Thailand and other Asian countries. Turmeric has the characteristic of being anti-fungal and anti-inflammatory (Lantz *et* al., 2005; Apisariyakul *et al*., 1995). Moreover, recent studies have also proven that turmeric exhibits anti-tumour, anti-microbial, anti-HIV, nematocidal and anti-oxidant properties which potentially act as an alternative and cheaper medicine source (Krup *et al*., 2013; Jayaprakasha *et al*., 2005; Araújo & Leon, 2001). Sookkongwaree *et al.,* (2006) had discovered that aqueous extracts from Zingiberaceae family (including turmeric) exhibited antiviral properties through viral protease inhibition. However the compound(s) responsible for the antiviral activity was not identified in the study. Based on this, the antiviral properties from turmeric extracts are speculated to be contributed by the presence of PIs and turmeric could exhibit PIs with protease inhibition activity. This could not be determined at the present moment as PIs and PI genes are yet to be discovered and studied from turmeric and the availability of molecular information on turmeric is limited. Hence, this research was aimed to answer the highlighted research problems with the following research objectives:

i) To isolate and characterize novel cysteine protease inhibitor (CYP) and Kunitz-type trypsin inhibitor (KTI) genes from leaves of *Curcuma longa*.
ii) To study the gene expression profile of CYP and KTI under methyl jasmonate treatment via RT-qPCR analysis
iii) To clone and optimise recombinant expression of CYP and KTI in bacterial-host system
iv) To optimise the purification of the recombinant CYP and KTI and screening of inhibitory activity and anti-pathogenicity potential
v) To generate a transcriptomic library of *Curcuma longa* via Illumina Hiseq platform

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