



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

FBSB 2016 38



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

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

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



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.)**

By

CHAN SEOW NENG

December 2016

Chairman: Noor Azmi Shaharuddin, PhD
Faculty: Biotechnology and Biomolecular Sciences

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-

thiogalactopyranoside (IPTG) used for the recombinant protein expression was determined at 37°C and 0.5 mM of IPTG for 2 hours induction. The recombinant *CypCl* was expressed as soluble protein while recombinant *CIKTI* was expressed as inclusion bodies. Recombinant *CypCl* was purified using immobilized metal affinity chromatography (IMAC) and anion-exchange chromatography while recombinant *CIKTI* was solubilized and refolded before purification by IMAC. The concentration of the purified recombinant *CypCl* obtained was 5 µg/µL and the refolded *CIKTI* 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.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

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

Oleh

CHAN SEOW NENG

Disember 2016

Pengerusi: Noor Azmi Shaharuddin, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

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, kebanyakan PI tumbuhan telah diciri dan diekspreskan 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 masalah 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 aktivitiya 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 *CIKTI*, telah diperolehi melalui kaedah *rapid amplification of cDNA ends* (RACE). Jujukan cDNA tersebut telah didepositkan dalam pangkalan data NCBI dengan nombor akses KF545951.1 (*CypCl*) dan KF889322.1 (*CIKTI*). Profil pengekspresan gen-gen *CypCl* dan *CIKTI* 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

terekspres sebagai protein larut manakala *CIKTI* rekombinan telah diekspreskan sebagai protein tidak larut (*inclusion bodies*). *CypCl* rekombinan telah dituliskan menggunakan *immobilized metal affinity chromatography* (IMAC) dan *anion-exchange chromatography* manakala *CIKTI* rekombinan telah disolubilisasikan dan melalui proses perlipatan semula sebelum dituliskan menggunakan IMAC. Kepekatan *CypCl* rekombinan yang dituliskan adalah sekitar 5 $\mu\text{g}/\mu\text{L}$ manakala *CIKTI* adalah sekitar 1 $\mu\text{g}/\mu\text{L}$. Protein rekombinan yang telah dituliskan tersebut telah menunjukkan aktiviti perencatan protease tetapi tidak menunjukkan potensi anti-patogen yang nyata berdasarkan kaedah penyingkapan 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 anotasi termasuk anotasi 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 dengan 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. Pengekspresan 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.

ACKNOWLEDGEMENTS

First of all, I would like to show my deepest gratitude and appreciation to my supervisor, Dr. Noor Azmi Shaharuddin, for his guidance and teaching throughout my graduate studies. He has been very helpful and is always able to assist me for the problems and challenges that I had faced while carrying out the lab work and experiments. I appreciate his patience and efforts spend in guiding me to the completion of my study and it has been an invaluable experience for me.

Secondly, sincerest thanks and deepest appreciation is expressed to my co-supervisors, Dr. Norliza Abu Bakar, Prof. Dr. Maziah Mahmood and Prof. Dr. Ho Chai Ling for accepting me as their co-supervised student and their precious insights while I carried out my research works. Special thanks to Dr. Norliza for allowing me to perform my labwork in her lab and I had learned a lot from her. I cannot thank her enough for her time and patience in guiding me. I would also like to thank UPM and MARDI for providing the facilities and workspace for me to complete all the works for my research.

Next, I would like to thank my parents and my siblings for their never ending love and support for me which is truly meaningful to me while I am undertaking my graduate studies. Special appreciation and thanks to those who had helped me a lot throughout my studies, especially Tan Swan, Rosazrinawati, and members of Proteomic Lab in Mardi.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Noor Azmi Shaharuddin, PhD

Senior Lecturer

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

Maziah Mahmood, PhD

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Member)

Ho Chai Ling, PhD

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Member)

ROBIAH BINTI YUNUS, PhD

Professor and Dean

School of Graduate Studies

Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No.: Chan Seow Neng (GS33884)

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of
Chairman of
Supervisory
Committee: _____

Signature: _____
Name of
Member of
Supervisory
Committee: _____

Signature: _____
Name of
Member of
Supervisory
Committee: _____

TABLE OF CONTENTS

ABSTRACT	Page
ABSTRAK	i
ACKNOWLEDGEMENTS	iii
APPROVAL	v
DECLARATION	vi
LIST OF TABLES	viii
LIST OF FIGURES	xvi
LIST OF APPENDICES	xxiii
LIST OF ABBREVIATIONS	xxix
LIST OF NOTATIONS	xxxii

CHAPTER

1	INTRODUCTION	1
2	LITERATURE REVIEW	
2.1	Plant Pathogenesis	3
2.1.1	General Plant Pathogens and Pest Insects	3
2.1.2	Protease in Plant Pathogenesis	4
2.1.2.1	General Properties of Protease	4
2.1.2.2	Role of Proteases in Plant Pathogenesis	4
2.1.3	Plant Defence Mechanism towards Pathogenesis	4
2.2	Protease Inhibitors	5
2.2.1	Classification of Protease Inhibitors	6
2.2.2	Protease Inhibitors in Plant Defence Mechanism	8
2.2.3	Overproduction of Protease Inhibitors in Plants	9
2.2.4	Different Modes of Protease Inhibition	12
2.3	Applications of Plant Protease Inhibitors in Industry	12
2.3.1	Candidate for Genetic Engineering in Phytoprotection	12
2.3.2	Drug Discovery	15
2.3.3	Food Preserving Industry	15
2.3.4	Resistance Development against Engineered Protection in Industry	16
2.4	Turmeric (<i>Curcuma longa</i>)	16
2.4.1	Medicinal Properties of <i>Curcuma longa</i>	17
2.4.2	Protease Inhibition by Zingiberaceae Family	18
2.4.2.1	Biochemical Compounds in Turmeric	18
2.4.2.2	Anti-pathogenic Agent in Turmeric	18
3	RESEARCH CHAPTER 1: IDENTIFICATION AND CHARACTERIZATION OF PROTEASE INHIBITOR GENES	
3.1	Introduction	19
3.2	Materials and Methods	19
3.2.1	Data Mining and Degenerate Primer Design	19
3.2.2	Planting of <i>Curcuma longa</i> , Treatment and Sampling	20
3.2.3	Total RNA Extraction	20
3.2.3.1	Cetyltrimethylammonium Bromide (CTAB)	20

3.2.3.2	RNAzol RT	21
3.2.3.3	Plant RNeasyMini Kit	21
3.2.4	Identification of Partial Protease Inhibitor cDNA	22
3.2.4.1	First-strand cDNA Synthesis	22
3.2.4.2	First Round Polymerase Chain Reaction (PCR)	22
3.2.4.3	Detection of PCR Products	22
3.2.4.4	Purification of PCR Products from Agarose Gel	23
3.2.4.5	Ligation	23
3.2.4.6	Transformation in OneShot TOP10 Competent Cells	23
3.2.4.7	Colony PCR Screening	24
3.2.4.8	Plasmid DNA Extraction	24
3.2.4.9	Plasmid DNA Digestion	25
3.2.4.10	DNA Sequencing	25
3.2.5	Identification of Novel Full-length PI cDNAs through RACE Method	25
3.2.5.1	Gene Specific Primers Design for RACE PCR	25
3.2.5.2	Generating RACE Ready cDNA	26
3.2.5.3	First round 5'- and 3'- RACE PCR	26
3.2.5.4	Generating the Full-length cDNA	27
3.2.6	Full-length Sequence Characterization	28
3.2.6.1	Sequence Analysis	28
3.2.6.2	Construction of Phylogenetic Tree	28
3.2.6.3	Three-dimensional (3D) Homology Modelling	28
3.3	Results and Discussions	29
3.3.1	Degenerate Primer Design	29
3.3.2	Comparisons of Different Total RNA Extraction Methods	30
3.3.3	Discovery of Novel Full-length PIs cDNA	32
3.3.3.1	Amplification of Novel Partial PIs cDNA	32
3.3.3.2	RACE: Generating the Novel Full-length Transcript	33
3.3.4	Sequence Analysis and Characterization of the Novel Full-length cDNA	35
3.3.4.1	Cysteine Protease Inhibitor, CYP	35
3.3.4.2	Kunitz Trypsin Inhibitor, KTI	43
3.4	Conclusion	47

4

RESEARCH CHAPTER 2: GENE EXPRESSION PROFILING OF *CypCl* AND *CIKT1* UNDER PLANT DEFENCE INDUCTION

4.1	Introduction	48
4.2	Materials and Methods	48
4.2.1	Plant Treatment, Sampling, Total RNA Extraction and First-strand cDNA Synthesis	48
4.2.2	Validation of DNA Primers in Real-time quantitative Polymerase Chain Reaction (RT-qPCR)	49

	4.2.2.1	Selection of Reference Gene Candidates and Specific DNA Primers Design for RT-qPCR in <i>Curcuma longa</i>	49
	4.2.2.2	Specificity and Efficiency Evaluation of Designed Primers	50
	4.2.3	Validation of Reference Genes for RT-qPCR in <i>Curcuma longa</i>	51
	4.2.4	Gene Expression Profiling of <i>CypCl</i> and <i>CIKTI</i> with RT-qPCR	52
4.3		Results and Discussions	52
	4.3.1	Preparations and Primer Design in <i>Curcuma longa</i> RT-qPCR	52
	4.3.1.1	Primer Specificity and Efficiency Tests	53
	4.3.2	Validations and Stability Ranking of Reference Gene Candidates	58
	4.3.3	<i>CypCl</i> and <i>CIKTI</i> Gene Expression Profiling with RT-qPCR	60
	4.3.3.1	<i>CypCl</i>	61
	4.3.3.2	<i>CIKTI</i>	62
4.4		Conclusion	63
5		RESEARCH CHAPTER 3: OPTIMISATION OF CLONING AND RECOMBINANT EXPRESSION OF CYP AND KTI IN BACTERIAL-HOST SYSTEM	
	5.1	Introduction	65
	5.2	Methods and Materials	65
	5.2.1	Construction of Recombinant PI Expression Ready Vector	65
	5.2.1.1	Primer Design for Recombinant Expression Studies	65
	5.2.1.2	Sampling, Total RNA Extraction and First-strand cDNA Synthesis	66
	5.2.1.3	First-round Polymerase Chain Reaction (PCR)	66
	5.2.1.4	Ligation into Cloning Vector, Transformation and Plasmid Extraction	66
	5.2.1.5	Double Digestion and Ligation in Expression Vector	67
	5.2.1.6	Confirmation of Insert in Expression Vector	67
	5.2.2	Recombinant Expression of PI	68
	5.2.2.1	Transformation of Expression Ready Vector in <i>E. coli</i> Expression Host	68
	5.2.2.2	Optimisation of Recombinant PI Expression Conditions in Small-Scale	68
	5.2.2.3	Determination of Protein Concentration Assay	69
	5.2.2.4	Detection of Protein Samples using SDS-PAGE	70
	5.2.2.5	Detection of His-tagged Recombinant PI using Western Blot	70

5.2.3	Large-scale Recombinant PI Expression	71
5.3	Results and Discussions	72
5.3.1	Construction and Confirmation of Recombinant PI Expression Ready Vector	72
5.3.2	First Round Recombinant PIs Expression Study	73
5.3.2.1	<i>CypCl</i>	74
5.3.2.2	<i>CIKTI</i>	78
5.3.3	Optimisation of Recombinant PIs Expression Ready Vector, Bacterial Host and Expression Conditions in <i>CIKTI</i>	81
5.3.3.1	Protein Toxicity	81
5.3.3.2	Disulphide Bond Formation	85
5.3.4	Large-scale Recombinant PI Expression	89
5.4	Conclusion	92

6 RESEARCH CHAPTER 4: OPTIMISATION OF RECOMBINANT PI PURIFICATION AND SCREENING OF PI INHIBITORY ACTIVITY AND ANTI-PATHOGENIC ACTIVITY

6.1	Introduction	93
6.2	Methods and Materials	93
6.2.1	Optimisation of Recombinant PI Purification	93
6.2.1.1	Column Preparation for Protein Purification (Gravity-flow)	93
6.2.1.2	Affinity Chromatography with Ni-NTA (Gravity-flow)	94
6.2.1.3	Column Preparation of Protein Purification (AKTAprime System)	94
6.2.1.4	Affinity Chromatography with Hi-Trap IMAC HP Column (AKTAprime System)	94
6.2.1.5	Buffer Exchange and Concentration of Purified Recombinant PIs	95
6.2.1.6	Anion-exchange Chromatography (AKTAprime System)	95
6.2.2	Inclusion Body Preparation and Manipulation	96
6.2.2.1	Washing and Solubilisation of Inclusion Bodies	96
6.2.2.2	Removal of solubilising agent and refolding	96
6.2.3	Detection of Purified Recombinant PIs using SDS-PAGE and Western Blot Analysis	97
6.2.4	Inhibitory Activity Assay	97
6.2.4.1	Determination of Inhibition Constant (K_i) and Mode of Inhibition of Recombinant <i>CypCl</i>	97
6.2.4.2	Recombinant <i>CIKTI</i> Inhibitory Activity Assay	98
6.2.5	Screening of Anti-Pathogenic Potential	98
6.3	Results and Discussions	99
6.3.1	Optimisation of Recombinant <i>CypCl</i> Purification	99
6.3.1.1	IMAC (Gravity-flow purification)	100

	6.3.1.2	IMAC (AKTAprime System)	103
	6.3.1.3	Anion-exchange Chromatography (AKTAprime System)	106
	6.3.2	Inhibitory Activity Study of Recombinant <i>CypCl</i>	109
	6.3.3	Inclusion Bodies Manipulation and Purification of Recombinant <i>ClKTI</i>	114
	6.3.4	Studies on Inhibition Activity of Recombinant <i>ClKTI</i>	121
	6.3.5	Screening of Recombinant PIs for Anti-Pathogenic Potential	122
	6.3.5.1	<i>CypCl</i>	123
	6.3.5.2	<i>ClKTI</i>	125
6.4		Conclusion	127
7		RESEARCH CHAPTER 5: GENERATION OF TRANSCRIPTOMIC LIBRARY OF <i>Curcuma longa</i>	
7.1		Introduction	128
7.2		Methods and Materials	128
	7.2.1	Plant Treatment, Sampling and Total RNA Extraction	128
	7.2.1.1	Total RNA Quality Assessment with Bioanalyzer	129
	7.2.2	RNA-sequencing with Next-generation Sequencing (NGS)	129
	7.2.2.1	Plate preparation for NGS	129
	7.2.2.2	RNA-sequencing	132
	7.2.3	Construction of Transcriptomic Library of <i>Curcuma longa</i> using Trinity software	132
	7.2.3.1	Pre-processing of Raw Transcriptomic Data	133
	7.2.3.2	<i>De novo</i> Assembly of Transcriptomic Data	133
	7.2.4	Functional Annotation	134
	7.2.5	Gene Expression Profiling in <i>Curcuma longa</i>	135
	7.2.5.1	Transcripts Expression Profile in Different Treatments	135
	7.2.5.2	Differential Expressed Gene (DEG) Studies in Methyl Jasmonate-treated Sample using EdgeR	136
7.3		Results and Discussions	136
	7.3.1	<i>Curcuma longa</i> Transcriptome Library Construction by Trinity	136
	7.3.1.1	Raw Data Pre-processing and Sequence Quality Control	136
	7.3.1.2	<i>De novo</i> Assembly	138
	7.3.2	Functional Annotation	139
	7.3.3	Gene Expression Profiling in <i>Curcuma longa</i>	142
	7.3.3.1	Transcript Expression Profiles of Each Treatment	142
	7.3.3.2	Differential Expressed Gene (DEG) Studies in Methyl Jasmonate-treated Sample	143
7.4		Conclusion	145

8	SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH	146
	REFERENCES	150
	APPENDICES	163
	BIODATA OF STUDENT	173
	LIST OF PUBLICATIONS	174



LIST OF TABLES

Table	Page
2.1 Examples of protease inhibitors' source and target protease based on their common family name and MEROPS family.	7
2.2 Examples of genetically engineered plants expressing plant PIs.	14
3.1 Sequences of the gene specific primer (GSP) used in performing 5' and 3' RACE according to the target protease inhibitor, namely cysteine protease inhibitor (CYP) and Kunitz trypsin inhibitor (KTI).	26
3.2 Sequences of the long distance primers designed from extreme of 5' and 3' RACE-PCR product used to generate full-length cDNA according to the target protease inhibitor, CYP and KTI.	27
3.3 Sequences of the degenerate primers designed from multiple sequence alignment by amino acid and cDNA information of other plant species for the first round PCR to isolate CYP and KTI.	30
3.4 Summary of the comparison results between the three different methods used for total RNA extraction in leave tissues of <i>Curcuma longa</i> .	32
4.1 List of designed primers for reference gene candidates and gene-of-interests.	50
4.2 Summary of the obtained results in the specific and efficiency test of designed RT-qPCR primers for reference gene candidates and genes of interests.	58
4.3 Summary of the calculated expression fold of the genes of interest in different methyl jasmonate treated to non-treated <i>Curcuma longa</i> tissues by using $\Delta\Delta CT$ method.	60
5.1 Sequences of primers designed from the open reading frame (ORF) of <i>CypCl</i> and <i>CIKTI</i> for construction of recombinant protein expression-ready vector. The restriction site for <i>Bam</i> HI and <i>Hind</i> III is underlined.	66
5.2 Labels of the centrifuge tube used and the optimisation of recombinant protein expression study based on different conditions such as incubation temperature, concentration of IPTG used and induction period.	68
5.3 The experiment layout for the construction of the standard curve by using BSA as standard compound in Lowry-like protein assay.	69

7.1	Number of the raw paired-reads obtained from NGS of Illumina Hiseq 2000 and reads after the quality assessments trimming.	138
7.2	Statistics from the <i>de novo</i> transcriptome assembly using trimmed reads combined from the three differently-treated <i>Curcuma longa</i> samples.	139
7.3	Summary of RNA-seq reads of each treatment mapped against the <i>de novo</i> transcriptome assembly of <i>Curcuma longa</i> .	143
8.1	Characterizations of PIs from <i>Curcuma longa</i> by using <i>in silico</i> bioinformatics tools.	146
8.2	Optimised parameters of recombinant expressions and purifications of <i>CypCl</i> and <i>ClKTI</i> .	147

LIST OF FIGURES

Figure	Page
2.1	11
The PI gene expression pathway in plants. The pathway includes the local and systemic signals as the elicitor of abscisic acid (ABA) inside the plant cell. ABA stimulates the breakdown membrane lipases into linolenic acid and activates the octadecanoid pathway. Volicitin, an oral secretion which are having a similar structure with linolenic acid also activates the octadecanoid pathway. The pathway produces jasmonic acid and leads to the mRNA expression of PIs (Adapted from Koiwa <i>et al.</i>, 1997).	
2.2	17
Turmeric (<i>Curcuma longa</i>) planted at the botanical garden of UPM. A) Turmeric plant. B) The rhizome of turmeric with an oblong shape and root-like branches.	
3.1	31
Comparison of total RNAs (1 µg) extracted from three different methods viewed on 1% agarose gel electrophoresis. The integrity of the total RNAs bands was different in comparison to the different methods used. 1) Modified CTAB method, 2) RNazol RT, 3) RNeasy Plant Mini Kit.	
3.2	33
The novel cDNA fragment amplified from degenerate PCR of A) cysteine protease inhibitor, CYP (500 bp) and B) Kunitz trypsin inhibitor, KTI (400 bp) viewed on 1% agarose gel respectively (M1: 1 kb DNA marker, M: 100 bp DNA marker).	
3.3	34
Detection of 5'-/3'- Rapid Amplification cDNA End (RACE) product generated by using SMARTer RACE kit. A1) 5'- RACE product for CYP, A2) 3'- RACE product for CYP, B) 5'- and 3'- RACE product for KTI. The PCR product was excised, purified and sent for DNA sequencing to determine the identity of the product. (M: 100 bp DNA marker)	
3.4	35
Detection of band formed from PCR product of full-length. A) CYP and B) KTI obtained from long distance PCR covering from 5'- to /3'- end by using primers designed from RACE products at size ~750 bp and ~850 bp respectively. (M: 100 bp DNA marker)	
3.5	37
Nucleotide and deduced amino acid sequences of the longest open reading frame (ORF) detected from the full-length CYP cDNA sequence. A signal peptide (from amino acid position 1-29th) as indicated by the horizontal arrow was predicted by using SignalP 3.0 program. The cleavage site is between amino acid 29 and 30 (as indicated with black arrow). Several motifs of phytocystatin were detected in the full-length sequence, such as i) one G residues towards N-terminal, ii) LARFAVEEHN, iii) QVVAG, iv) PW dipeptide and v) SNSL. The respective motifs had been boxed.	

3.6	Graphical summary of BLAST results of the deduced amino acids of <i>CypCl</i> on conserved domains. It showed that several conserved cystatin superfamily domains are detected on the sequence suggesting a novel putative phytocystatin isolated from <i>Curcuma longa</i> .	38
3.7	Phylogenetic tree generated with phytocystatins containing C-terminal extensions by using neighbour-joining method with 100 bootstrapping. <i>CypCl</i> shown to be related to phytocystatin from <i>Elaeis guineensis</i> (African oil palm).	38
3.8	Structural model of the identified <i>CypCl</i> . Common motifs of cystatin such as the LARFAVEEHN (red), QVVAG (green) and PW (yellow) were detected from the amino acid sequence. The tertiary structure was modelled by using phytocystatin structure acquired from <i>Sesamum indicum</i> as model template.	40
3.9	Multiple sequence alignments of the deduced amino acids of the full-length sequence of <i>CypCl</i> (indicated with black arrow) with other phytocystatin sequences from other plants obtained from the GenBank database in the NCBI website.	42
3.10	A full-length sequence of <i>ClKTI</i> with deduced amino acid sequence. A start codon, ATG, is located at nucleotide position 3 while a stop codon, TAG, is located at position 641-643. The motif of Kunitz trypsin inhibitor (KTI) ([L,I,V,M]-X-D-X ₂ -G-X ₂ [L,I,V,M]-X ₅ -Y-X-[L,I,V,M]) and the reactive site motif (G/E-I-S) were detected as boxed. Signal peptide was detected on the sequence, which spanned by a length of 24 amino acids as indicated by the horizontal arrow and the calculated cut-off point is between the 24 th and 25 th amino acids (AEA-TS), as indicated by the vertical arrow.	44
3.11	Graphical summary of BLAST results of the deduced amino acids of <i>CypCl</i> on conserved domains. It shows that several conserved cystatin superfamily domains are detected on the sequence suggesting a novel putative phytocystatin isolated from <i>Curcuma longa</i> .	45
3.12	Phylogenetic tree constructed using neighbour-joining method with 100 bootstrapping using deduced amino acids sequence of <i>ClKTI</i> (accession no: AHJ25677.1) with other KTI sequences from different plant species. <i>ClKTI</i> shows the closest evolutionary relationship with KTI belonging to <i>Theobroma cacao</i> .	45

3.13	Ribbon representation of the predicted 3D protein model of <i>CIKTI</i> based on homology modelling. Cysteine residues were represented in green ball-and-stick and disulphide bridges were indicated in lines and labelled. The theoretical <i>CIKTI</i> tri-dimensional protein structure was modelled by using a trypsin inhibitor from <i>Murraya koenigii</i> as model template.	47
4.1	Specificity test of the designed specific RT-qPCR primers designed on 1% agarose gel electrophoresis by using the amplified PCR products. A) The specificity test of <i>CypCl</i> primers in the different <i>Curcuma longa</i> tissues. (M = 100 bp DNA marker, 1 = Flower, 2 = Basal Stem, 3 = Stem, 4 = Rhizome, 5 = Root). B) The specificity test of <i>CIKTI</i> and three other reference gene candidates in the different <i>Curcuma longa</i> tissues. (M = 100 bp DNA marker, 1 = <i>CIKTI</i> , 2 = <i>Actin</i> , 3 = α -tubulin, 4 = <i>Ubox</i>). The amplified PCR products of the different designed primers of the respective <i>Curcuma longa</i> tissues showed the formation of single band on the 1% agarose gel, indicating specificity of the designed primers.	54
4.2	Specificity validation of the designed RT-qPCR primers through melting curve analysis. Based on the resulted melting curve, only a single peak was detected from the respective targets and this indicated that the designed primers were specific with a single amplicon. (A = <i>Actin</i> , B = <i>Alpha-tubulin</i> , C = <i>Ubiquitin</i> , D = <i>CypCl</i> , E = <i>CIKTI</i>)	56
4.3	Standard curve analysis for the efficiency test of the designed RT-qPCR primers. Based on the resulted standard curve, the efficiency of the designed primers and R^2 value of the plot were within the acceptable range in which a 90% - 110% for the efficiencies and 0.98 - 0.99 for R^2 value. (A = <i>Actin</i> , B = <i>Alpha-tubulin</i> , C = <i>Ubiquitin</i> , D = <i>CypCl</i> , E = <i>CIKTI</i>)	57
4.4	Expression stability of the reference gene candidates and their stability ranking generated by RefFinder. The quantification cycle value, Cq, of the three reference genes expressed in five different tissues, namely flower, stem, basal stem, rhizome and root in all treatment condition was used in the analysis. A) RefFinder incorporated the different software and algorithms to calculate the expression stability and ranked them B) A comprehensive result generated by RefFinder and <i>actin</i> was ranked as the most stable reference gene.	59
4.5	Normalized fold expression of <i>CypCl</i> and <i>CIKTI</i> in methyl-jasmonate (MJ) treated and non-treated (NT) tissues by RT-qPCR using <i>actin</i> as reference gene for normalization. Generally, the expression of <i>CypCl</i> was similar between MJ treated and NT tissues while the expression of <i>CIKTI</i> was higher in MJ treated as compared to NT tissues.	61

5.1	Colony PCR analysis on the presences of inserted ORF in the expression vector. A single intact band at the expected size was detected from the PCR colony product using the specific <i>CypCl</i> and <i>CLKTI</i> primer and this act as the preliminary indication of the presence of the insert. (M: 1 kb DNA marker, 1 – 7: Transformed colonies)	73
5.2	Restriction endonuclease analysis by <i>Bam</i> HI and <i>Hind</i> III on the presences of the inserted ORF in the expression vector. Intact band at the expected size was detected on the 1% agarose gel after the double digestion and was indicative of the presences of the insert. (M: 100 bp DNA marker, 1: <i>CypCl</i> , 2: <i>CLKTI</i>)	73
5.3	SDS-PAGE analysis comparing recombinant proteins expressed in the supernatant of BL21(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of <i>CypCl</i> (on the right). The results indicated that recombinant <i>CypCl</i> was successfully expressed at the size ~30 kDa from pRSETA as the band intensity is higher compared to the control samples (empty pRSETA). (M: Marker; Xi: Uninduced)	76
5.4	Western blot analysis in detecting recombinant <i>CypCl</i> proteins in comparison between the supernatant obtained from BL21(DE3) cells induced with 0.5 mM IPTG containing empty pRSETA (on the left) and pRSETA containing ORF of <i>CypCl</i> (on the right). The results confirmed the results from SDS-PAGE where soluble recombinant <i>CypCl</i> protein was expressed under the different incubation temperatures. (M: Marker; Xi: Uninduced)	76
5.5	SDS-PAGE analysis comparing recombinant proteins expressed in pellets of BL21(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of <i>CypCl</i> (on the right). Recombinant protein was found expressed as inclusion bodies in the pellet at the size ~26 kDa from pRSETA and the band intensity is higher as compared to the control samples (empty pRSETA). (M: Marker; Xi: Uninduced)	77
5.6	Western blot analysis in detecting expressed recombinant proteins in comparison between the pellet obtained from BL21(DE3) cells induced with 0.5 mM IPTG containing empty pRSETA (on the left) and pRSETA containing ORF of <i>CypCl</i> (on the right). The results indicated that there was recombinant proteins expressed as inclusion bodies as predicted from the SDS-PAGE analysis. (M: Marker; Xi: Uninduced)	77

- 5.7 SDS-PAGE analysis comparing recombinant proteins expressed in the supernatant of BL21(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of *CIKTI* (on the right). The results indicated that recombinant protein was expressed at the size ~30 kDa from pRSETA as the band intensity is higher compared to the control samples (empty pRSETA). (M: Marker; Xi: Uninduced) 79
- 5.8 Western blot analysis in detecting recombinant *CIKTI* proteins in comparison between the induced supernatant obtained from BL21(DE3) cells containing empty pRSETA (on the left) and pRSETA containing ORF of *CIKTI* (on the right). The results showed that only low amount of recombinant protein was present in the induced supernatant sample as faint band was detected on the membrane. The recombinant protein could be a contamination of recombinant *CIKTI* from pellet samples. The results were the same for the different incubation temperature. (M: Marker; Xi: Uninduced) 79
- 5.9 SDS-PAGE analysis comparing recombinant proteins expressed in the pellet of BL21(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of *CIKTI* (on the right). The results indicated that recombinant *CIKTI* was expressed at the size ~30 kDa from pRSETA as the band intensity is higher compared to the control samples (empty pRSETA) and the size matched the expected size. (M: Marker; Xi: Uninduced) 80
- 5.10 Western blot analysis in detecting recombinant *CIKTI* proteins in comparison between the induced supernatant obtained from BL21(DE3) cells containing empty pRSETA (on the left) and pRSETA containing ORF of *CIKTI* (on the right). The results showed that the size of the band matched the expected size of recombinant *CIKTI*. Majority of the expressed recombinant *CIKTI* was detected in induced pellet sample as the band was in higher intensity compared to the band from the supernatant sample. The results were the same for the different incubation temperature. (M: Marker; Xi: Uninduced) 80
- 5.11 SDS-PAGE analysis comparing recombinant proteins expressed in the supernatant of C43(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of *CIKTI* (on the right). The results showed that there was not much different detected between the protein profiles of both samples. (M: Marker; Xi: Uninduced) 83

5.12	Western blot analysis comparing recombinant proteins expressed in the supernatant of C43(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of <i>CIKTI</i> (on the right). There were no bands detected from the protein profiles of both samples and this indicated that no recombinant protein was expressed in the supernatant samples of both conditions in the C43(DE3) host. (M: Marker; Xi: Uninduced)	83
5.13	SDS-PAGE analysis comparing recombinant proteins expressed in the pellet of C43(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of <i>CIKTI</i> (on the right). Similarly to the results from supernatant, there was not much different detected between the protein profiles of both samples. (M: Marker; Xi: Uninduced)	84
5.14	Western blot analysis comparing recombinant proteins expressed in the pellet of C43(DE3) cells induced with 0.5 mM IPTG transformed with empty pRSETA (on the left) and pRSETA containing ORF of <i>CIKTI</i> (on the right). There were no bands detected from the protein profiles of both samples and this indicated that no recombinant protein was expressed in the pellet samples of both conditions in the C43(DE3) host. (M: Marker; Xi: Uninduced)	84
5.15	SDS-PAGE analysis comparing recombinant proteins expressed in the supernatant (left) and pellet (right) samples of Origami cells induced with 0.5 mM IPTG transformed with pET32b containing ORF of <i>CIKTI</i> incubated at 20°C. The results showed that the recombinant <i>CIKTI</i> could be formed as inclusion bodies as higher band intensity was detected in pellet samples. (M: Marker; Xi: Uninduced; 1,2,3,4: Samples)	87
5.16	Western blot analysis comparing recombinant proteins expressed in the supernatant (left) and pellet (right) samples of Origami cells induced with 0.5mM IPTG, transformed with pET32b containing ORF of <i>CIKTI</i> incubated at 20°C. The results showed that large amount of the expressed recombinant <i>CIKTI</i> was formed as inclusion bodies as intense band was detected in pellet samples at the size of ~35 kDa. (M: Marker; Xi: Uninduced; 1,2,3,4: Samples)	87
5.17	SDS-PAGE analysis comparing recombinant proteins expressed in the supernatant (left) and pellet (right) samples of BL21(DE3) induced with 0.5 mM IPTG, transformed with pET32b containing ORF of <i>CIKTI</i> and incubated different temperatures. The results showed that the recombinant <i>CIKTI</i> could be formed as inclusion bodies at the size ~30 kDa as higher band intensity was detected in pellet samples. (M: Marker; Xi: Uninduced)	88

5.18	Western blot analysis comparing recombinant proteins expressed in the supernatant (left) and pellet (right) samples of BL21(DE3) induced with 0.5 mM IPTG, transformed with pET32b containing ORF of <i>CIKTI</i> and incubated different temperatures. The results showed that a large amount of recombinant <i>CIKTI</i> were formed as inclusion bodies at the size ~30 kDa with 37°C incubation temperature having the highest intensity. (M: Marker; Xi: Uninduced)	88
5.19	SDS-PAGE analysis comparing the recombinant proteins expressed in the supernatant (left) and pellet (right) in the large scale recombinant expression of <i>CypCl</i> . The results showed that high intensity bands were detected at the expected size of ~30 kDa suggesting the presence of <i>CypCl</i> . (M: Marker; 1,2,3,4: Samples)	90
5.20	Western blot analysis comparing the recombinant proteins expressed in the supernatant (left) and pellet (right) in the large scale recombinant expression of <i>CypCl</i> . The results confirmed that soluble recombinant <i>CypCl</i> was successfully expressed at the expected size of ~30 kDa from the supernatant samples. (M: Marker; 1,2,3,4: Samples)	90
5.21	SDS-PAGE analysis comparing the recombinant proteins expressed in the supernatant (left) and pellet (right) in the large scale recombinant expression of <i>CIKTI</i> . The results showed that high intensity bands were detected at the expected size of ~30 kDa suggesting the presence of <i>CIKTI</i> . (M: Marker; 1,2,3: Samples)	91
5.22	Western blot analysis comparing the recombinant proteins expressed in the supernatant (left) and pellet (right) in the large scale recombinant expression of <i>CIKTI</i> . The results confirmed that the inclusion bodies of recombinant <i>CIKTI</i> were successfully expressed at the expected size of ~30 kDa from the pellet samples. (M: Marker; 1,2,3: Samples)	91
6.1	SDS-PAGE analysis on the fractions obtained from the different stages of recombinant <i>CypCl</i> purification by immobilized metal affinity chromatography (IMAC). The results (Lane 6 – 9) indicated that <i>CypCl</i> was successfully purified from the supernatant but there were also presences of contaminant proteins other than <i>CypCl</i> eluted. (Lane M: Marker; Lane 1: Flow-through; Lane 2: Wash 1; Lane 3: Wash 2; Lane 4: Wash 7; Lane 5: Wash 8; Lane 6: Elution 1; Lane 7: Elution 2; Lane 8: Elution 7; Lane 9: Elution 8)	102

6.2	Western blot analysis on the fractions obtained from the different stages of recombinant <i>CypCl</i> purification by immobilized metal affinity chromatography (IMAC). The results (Lane 6 – 9) indicated that <i>CypCl</i> was successfully purified from the supernatant. (Lane M: Marker; Lane 1: Flow-through; Lane 2: Wash 1; Lane 3: Wash 2; Lane 4: Wash 7; Lane 5: Wash 8; Lane 6: Elution 1; Lane 7: Elution 2; Lane 8: Elution 7; Lane 9: Elution 8)	102
6.3	Immobilized metal affinity chromatography (IMAC) purification chromatogram of the recombinant <i>CypCl</i> by using the pre-packed HiTrap IMAC HP column paired with the AKTAprime system. The results showed that a single peak was detected when the column was eluted with the elution buffer, which indicated the presence of the purified recombinant <i>CypCl</i> . The blue line represents the UV absorbance of protein eluted out from the column; the red line represents the conductivity in the column; the green line represents the concentration of elution buffer in the column.	104
6.4	SDS-PAGE analysis of the fractions containing the purified recombinant <i>CypCl</i> obtained from the elution step in immobilized metal affinity chromatography (IMAC) by using pre-packed HiTrap HP Column paired with AKTAprime System. The results showed that a single high intensity band was detected at the expected molecular weight of <i>CypCl</i> . (M: Marker; F1 – F7: Fractions)	105
6.5	Western blot analysis in detecting the presence of purified recombinant <i>CypCl</i> in the collected elution fractions obtained in immobilized metal affinity chromatography (IMAC) by using pre-packed HiTrap HP Column paired with AKTAprime System. The results showed that a single high intensity band was detected at the expected molecular weight of <i>CypCl</i> . (M: Marker; F1 – F7: Fractions)	105
6.6	Anion-exchange chromatography purification chromatogram of the recombinant <i>CypCl</i> by using the pre-packed HiTrap Q XL HP column paired with the AKTAprime system. The results showed that a single peak was detected when the column was eluted with increasing in gradient-wise of the 1 M NaCl solution, which indicated the presence of the purified recombinant <i>CypCl</i> . The blue line represents the UV absorbance of protein eluted out from the column; the red line represents the conductivity in the column; the green line represents the concentration of elution buffer in the column.	107

6.7	SDS-PAGE analysis of the fractions containing the purified recombinant <i>CypCl</i> obtained from the elution step in anion-exchange chromatography by using pre-packed HiTrap Q XL column paired with AKTAprime System. The results showed that a single high intensity band was detected at the expected molecular weight of <i>CypCl</i> and only faint traces of contaminant proteins were present. (M: Marker; F1 – F7: Fractions)	108
6.8	Western blot analysis in detecting the presence of purified recombinant <i>CypCl</i> in the collected elution fractions obtained from anion-exchange chromatography by using pre-packed HiTrap Q XL Column paired with AKTAprime System. The results showed that a single high intensity band was detected at the expected molecular weight of <i>CypCl</i> . (M: Marker; F1 – F7: Fractions)	108
6.9	Inhibition of papain activity by recombinant <i>CypCl</i> . 20 μ M of papain was incubated with various concentrations of recombinant <i>CypCl</i> and the residual papain activity was determined by the addition of substrate, BANA, and colour development by p-DMCA. A) The colour development in solution containing increasing amount of recombinant <i>CypCl</i> . B) Graph showing the residual papain activity (%) detected in the increasing concentration of recombinant <i>CypCl</i> .	110
6.10	Lineweaver-Burk plot of papain inhibition assays inhibited by purified recombinant <i>CypCl</i> . The papain activity was measured in the papain assay with increasing amount of BANA concentrations, conducted with the presences of 50 μ g of recombinant <i>CypCl</i> and without any inhibitor (replaced by elution buffer). From the constructed plot, it indicated that recombinant <i>CypCl</i> was a non-competitive inhibitor.	112
6.11	Dixon plot of the inhibition of papain activity by increasing amount of recombinant <i>CypCl</i> . The assay was conducted with 20 μ M of papain in increasing concentration of recombinant <i>CypCl</i> , E^{-08} M, in two different concentration of substrate (1 mM and 2 mM). From the Dixon plot, the K_i was determined at the meeting point of the two plotted lines on the x-axis. The obtained K_i value of recombinant <i>CypCl</i> was $2.33 \pm 0.01 \times 10^{-8}$ M or 23 nM.	113
6.12	SDS-PAGE analysis of the supernatant and pellet samples acquired from the inclusion bodies washing and solubilisation step of recombinant <i>CIKTI</i> . It can be observed in the supernatant samples that most of the impurities were removed gradually from the washing step and increased the purity of the pellet. It can be observed in the SS sample that the most of the recombinant <i>CIKTI</i> was solubilised. M: Marker; SS: Solubilised supernatant; SP: Solubilised pellet; S: Supernatant; P: Pellet)	115

6.13	Western blot analysis of the supernatant and pellet samples acquired from the washing and solubilisation step of recombinant <i>CIKTI</i> inclusion bodies. The solubilised recombinant <i>CIKTI</i> was detected in the SS sample and most of the inclusion bodies were solubilised as only a faint band was detected in the SP sample. M: Marker; SS: Solubilised supernatant; SP: Solubilised pellet; S: Supernatant; P: Pellet)	115
6.14	Chromatogram of the refolding process of solubilised recombinant <i>CIKTI</i> by using on-column procedures with pre-packed HiTrap IMAC column paired with the AKTAprime system. The chromatogram showing the refolding process where contaminant proteins were washed out during the washing step and the refolding of the solubilised recombinant <i>CIKTI</i> was completed with the increasing refolding buffer. The blue line represents the UV absorbance of protein eluted out from the column; the red line represents the conductivity in the column; the green line represents the concentration of refolding buffer in the column.	118
6.15	Chromatogram of elution of the refolded recombinant <i>CIKTI</i> using on-column procedures with pre-packed HiTrap IMAC column paired with the AKTAprime system. The chromatogram indicated that the refolded recombinant <i>CIKTI</i> was purified and eluted out during the elution with 100% concentration of elution buffer containing 250 mM imidazole. The blue line represents the UV absorbance of protein eluted out from the column; the red line represents the conductivity in the column; the green line represents the concentration of elution buffer in the column.	119
6.16	SDS-PAGE analysis of the fractions containing the purified recombinant <i>CIKTI</i> after refolding and eluted from the pre-packed HiTrap HP IMAC column paired with AKTAprime System. The results showed that a high intensity band was detected at the expected molecular weight of <i>CIKTI</i> and there were some faint traces of contaminant proteins present. (M: Marker; F1 – F8: Fractions)	120
6.17	Western-blot analysis of the fractions containing the purified recombinant <i>CIKTI</i> after refolding and eluted from the pre-packed HiTrap HP IMAC column paired with AKTAprime System. The results confirmed the presence of recombinant <i>CIKTI</i> was purified as high intensity band was detected at the expected molecular weight of <i>CIKTI</i> . Nonetheless, faint traces of contaminant proteins were also detected to be eluted together with the refolded recombinant <i>CIKTI</i> . (M: Marker; F1 – F8: Fractions)	120
6.18	The residual trypsin activity in the increasing volume of refolded recombinant <i>CIKTI</i> (1 µg/µL). The amount of bovine pancreas inhibitor used in the test was 100 µg and its activity was expected to be completely inhibited at ~80 µg of <i>CIKTI</i> .	122

6.19	Anti-bacterial screening using recombinant <i>CypCl</i> against two different <i>Pseudomonas</i> sp. 1) 20 μ L of ampicillin solution (5 mg/mL), 2) 20 μ L of elution buffer from IEX chromatography, 3) 20 μ L of <i>CypCl</i> (1 μ g/ μ L) and 4) 20 μ L of <i>CypCl</i> (5 μ g/ μ L).	123
6.20	Anti-fungal screening by using 20 μ L of different amount of recombinant <i>CypCl</i> (0 = control, 5 = 25 μ g, 10 = 50 μ g and 20 = 100 μ g) against the A) <i>Colletotrichum gloeosporioides</i> , B) <i>Pestalotiopsis theae</i> , C) <i>Pyricularia oryzae</i> and D) <i>Fusarium solani</i> .	124
6.21	Anti-pathogenic screening by the solubilised and refolded recombinant <i>ClKTI</i> . A) Anti-bacterial potential screening against <i>Pseudomonas</i> sp. 1) 20 μ L of 5 mg/mL ampicillin, 2) 20 μ L of IMAC elution buffer and 3) 20 μ L of refolded recombinant <i>ClKTI</i> (1 μ g/ μ L). Anti-fungal potential screening conducted using 50 μ L of <i>ClKTI</i> against B) <i>Fusarium solani</i> and C) <i>Pyricularia oryzae</i> where the 'X' indicated 20 μ L of IMAC elution buffer as control and 'S' indicated 20 μ L of refolded <i>ClKTI</i> .	126
7.1	Overall graph representation of the number of RNA-seq reads before and after the quality assessment trimming in the differently treated samples. (W – wound-treated, MJ – methyl jasmonate-treated, C – control)	138
7.2	The number of assembled transcripts from the <i>Curcuma longa</i> samples having sequence homologies with the entries from different queried databases. The number of unique protein entries identified in database of UniProt, Pfam and GO was 13,820, 3,905 and 10,532 respectively.	141
7.3	The percentage of assembled transcripts in <i>Curcuma longa</i> annotated by GO term categorized into different functional categories namely biological process, cellular component and molecular function.	142
7.4	Volcano plot visualising the differential expression in methyl jasmonate-treated samples where each dot representing a transcript and significant differential expression were represented in red dots.	144

LIST OF APPENDICES

Appendix		Page
A1	IUPAC ambiguity codes for degenerate primer design.	163
A2	Calculation of the expression fold of <i>CIKTI</i> in comparison of methyl-jasmonate treated and non-treated in different <i>Curcuma longa</i> tissues by real-time qPCR using $\Delta\Delta CT$ method.	164
A3	Inhibition of papain activity by using different concentration of purified recombinant <i>CypCl</i> . Absorbance reading was produced from the residual papain activity in the presences of difference concentration of recombinant <i>CypCl</i> by using BANA as substrate and p-DMCA to develop the colour.	165
A4	Inhibition activity on bovine pancreas trypsin by using different concentrations of purified recombinant <i>CIKTI</i> . The changes in absorbance value of the solution with and without the presence of the various <i>CIKTI</i> concentrations were recorded and the inhibitory activities were calculated.	166
B1	Blue-white colony screening where ligation product of pGEM-T vector with the target cDNA was transformed into TOP10 competent cells and spread on LB-ampicillin agar plate. White colony indicates successfully transformed colony while blue colony indicates unsuccessful transformant.	167
B2	Comparison between recombinant proteins profiles induced by two different concentrations of IPTG for the recombinant expression of <i>CypCl</i> using pRSETA vector transformed in BL21DE3 competent cells. The band formed by the recombinant <i>CypCl</i> was as indicated by the black arrow. (Xi: Uninduced; M: Marker)	168
B3	The unscaled chromatogram of total purification of recombinant <i>CypCl</i> by using HiTrap IMAC column paired with the AKTAprime system.	169
C1	The partial cDNA sequences of alpha tubulin and ubox acquired from other PCR reactions involving <i>Curcuma longa</i> . These sequences were used in the primer design of reference gene candidates.	170
D1	Recipe for buffers	171

LIST OF ABBREVIATIONS

3D	Three-dimensional
ABA	Absciscic acid
BAEE	Na-benzoyl-L-arginine ethyl ester
BANA	N-benzoyl-L-arginine-2-naphthylamide
BBi	Bowman Birk Inhibitor
BLAST	Basic Local Alignment Search Tools
BSA	Bovine Serum Albumin
Bt	<i>Bacillus thuringiensis</i>
cAMP	Cyclic adenosinemonophosphate
cDNA	Complementary-deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
CYP	Cysteine protease inhibitor
DEG	Differential expressed gene
dH ₂ O	Distilled H ₂ O
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
<i>et al.</i>	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

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

LIST OF NOTATIONS

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

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).

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

REFERENCES

- Abe, M., Abe, K., Iwabuchi, K., Domoto, C., & Arai, S. (1994). Corn cystatin I expressed in *Escherichia coli*: Investigation of its inhibitory profile and occurrence in corn kernels. *Journal of Biochemistry* 492:488–492.
- Alfano, J. R., & Collmer, A. (2001). Mechanisms of bacterial pathogenesis in plants: Familiar foes in a foreign kingdom. In *Principles of Bacterial Pathogenesis*, ed. E.A. Groisman, pp. 179–226. Missouri: Academic Press.
- Almeida Barros, B., Da Silva, W. G., Moreira, M. A., & De Barros, E. G. (2012). In silico characterization and expression analysis of the multigene family encoding the Bowman-Birk protease inhibitor in soybean. *Molecular Biology Reports* 39(1):327–334.
- Amalraj, A., Pius, A., Gopi, S., & Gopi, S. (2016). Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives – A review. *Journal of Traditional and Complementary Medicine* In press. [doi:[10.1016/j.jtcme.2016.05.005](https://doi.org/10.1016/j.jtcme.2016.05.005)].
- Ammon, H. P., Anazodo, M. I., Safayhi, H., Dhawan, B. N., & Srimal, R. C. (1992). Curcumin: A potent inhibitor of leukotriene B₄ formation in rat peritoneal polymorphonuclear neutrophils (PMNL). *Planta medica* 58(2):226.
- Andersen, C. L., Jensen, J. L., & Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* 64(15):5245–5250.
- Apisariyakul, a, Vanittanakom, N., & Buddhasukh, D. (1995). Antifungal activity of turmeric oil extracted from *Curcuma longa* (Zingiberaceae). *Journal of Ethnopharmacology* 49(3):163–169.
- Araújo, C. C., & Leon, L. L. (2001). Biological activities of *Curcuma longa* L. *Memórias do Instituto Oswaldo Cruz* 96(5):723–728.
- Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., et al. (2000). Gene Ontology: Tool for the unification of biology. *Nature Genetics* 25(1):25–29.
- Balouiri, M., Sadiki, M., & Ibensouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* 6(2):71–79.
- Bangrak, P., & Chotigeat, W. (2011). Molecular cloning and biochemical characterization of a novel cystatin from *Hevea* rubber latex. *Plant Physiology and Biochemistry* 49(3):244–250.
- Benchabane, M., Goulet, M.C., Dallaire, C., Côté, P.L., & Michaud, D. (2008). Hybrid protease inhibitors for pest and pathogen control--a functional cost for the fusion partners? *Plant Physiology and Biochemistry* 46(7):701–708.

- Benchabane, M., Schluter, U., Vorster, J., Goulet, M. C., & Michaud, D. (2010). Plant cystatins. *Biochimie* 92(11):1657–1666.
- Bergey, D. R., Howe, G. A., & Ryan, C. A. (1996). Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proceedings of the National Academy of Sciences of the United States of America* 93(22): 12053–12058.
- Bhat, S. V., Amin, T., & Nazir, S. (2016). Biological Activities of Turmeric (*Curcuma longa* Linn.) - An Overview. *BMR Microbiology* 2(1):1-5.
- Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., et al. (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic acids research* 42(Web Server issue):252–258.
- Bijina, B., Chellappan, S., Krishna, J. G., Basheer, S. M., Elyas, K. K., Bahkali, A. H., & Chandrasekaran, M. (2011). Protease inhibitor from *Moringa oleifera* with potential for use as therapeutic drug and as seafood preservative. *Saudi Journal of Biological Sciences* 18(3):273–281.
- Birk, Y. (2010). *Plant Protease Inhibitors: Significance in Nutrition, Plant Protection, Cancer Prevention and Genetic Engineering*. Berlin:Springer.
- Birnbaum, S., & Bailey, J. E. (1991). Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant *Escherichia coli*. *Biotechnology and Bioengineering* 37(8):736–745.
- Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., et al. (1988). The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *The EMBO Journal* 7(8): 2593–2599.
- Botella, M. a, Xu, Y., Prabha, T. N., Zhao, Y., Narasimhan, M. L., Wilson, K. a, Nielsen, S. S., et al. (1996). Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. *Plant Physiology* 112:1201–1210.
- Brady, R. L. (2003). *Plant Protease Inhibitors: Significance in Nutrition, Plant Protection, Cancer Prevention and Genetic Engineering*. *Phytochemistry* (Vol. 64). Berlin, Heidelberg, NY: Springer-Verlag.
- Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., et al. (2009). The MIQE Guidelines : Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* 55(4):611–622.
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST plus: architecture and applications. *BMC Bioinformatics* 10(421):1.

- Chen, M. (2008). Inducible direct plant defense against insect herbivores : A review. *Insect Science* 15:101–114.
- Cheung, A. H. K., Wong, J. H., & Ng, T. B. (2009). Trypsin-chymotrypsin inhibitors from *Vigna mungo* seeds. *Protein and peptide letters* 16(3):277–284.
- Chomczynski, P., Wilfinger, W., Kennedy, A., Rymaszewski, M., & Mackey, K. (2010). RNAzol®RT : A new single-step method for isolation of RNA. *Nature Publishing Group* 7(12):an4–an5.
- Chopra, R., Burow, G., Farmer, A., Mudge, J., Simpson, C. E., & Burow, M. D. (2014). Comparisons of de novo transcriptome assemblers in diploid and polyploid species using peanut (*Arachis* spp.) RNA-Seq data. *PLoS one* 9(12), e115055.
- Christou, P., Capell, T., Kohli, A., Gatehouse, J. A., & Gatehouse, A. M. R. (2006). Recent developments and future prospects in insect pest control in transgenic crops. *Trends in plant science* 11(6):302–308.
- Clarke, K., Yang, Y., Marsh, R., Xie, L. L., & Zhang, K. K. (2013). Comparative analysis of de novo transcriptome assembly. *Science China Life Sciences* 56(2):156–162.
- Corchero, J. L., & Villaverde, A. (1998). Plasmid maintenance in *Escherichia coli* recombinant cultures is dramatically, steadily, and specifically influenced by features of the encoded proteins. *Biotechnology and Bioengineering* 58(6):625–632.
- Cox, M. P., Peterson, D. A., & Biggs, P. J. (2010). SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* 11(1):485.
- Cruz, A. C. B., Massena, F. S., Migliolo, L., Macedo, L. L. P., Monteiro, N. K. V., Oliveira, A. S., Macedo, F. P., *et al.* (2013). Bioinsecticidal activity of a novel Kunitz trypsin inhibitor from Catanduva (*Piptadenia moniliformis*) seeds. *Plant Physiology and Biochemistry* 70:61–68.
- Cseke, L., & Kirakosyan, A. (2016). *Handbook of Molecular and Cellular Methods in Biology and Medicine, Third Edition*.:CRC Press.
- Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., *et al.* (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* 13:414–430.
- Demidenko, N. V., Logacheva, M. D., & Penin, A. A. (2011). Selection and validation of reference genes for quantitative real-time PCR in buckwheat (*Fagopyrum esculentum*) based on transcriptome sequence data. *PLoS ONE* 6(5):1–9.
- Derman, a I., Prinz, W. a, Belin, D., & Beckwith, J. (1993). Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science* 262(5140):1744–1747.

- Dixon, M. (1972). The graphical determination of K_m and K_i . *The Biochemical Journal* 129(1):197–202.
- Doherty, A. J., Connolly, B. A., & Worrall, A. F. (1993). Overproduction of the toxic protein, bovine pancreatic DNaseI, in *Escherichia coli* using a tightly controlled T7-promoter-based vector. *Gene* 136(1-2):337–340.
- Dong, L., Lv, L.B., & Lai, R. (2012). Molecular cloning of *Tupaia belangeri chinensis* neuropeptide Y and homology comparison with other analogues from primates. *Zoological Research* 33:75–78.
- Dumon-Seignovert, L., Cariot, G., & Vuillard, L. (2004). The toxicity of recombinant proteins in *Escherichia coli*: A comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). *Protein Expression and Purification* 37(1):203–206.
- Dunaevskii, Y. E., Matveeva, A. R., Fatkhullina, G. N., Belyakova, G. A., Kolomiets, T. M., Kovalenko, E. D., & Belozersky, M. A. (2008). Extracellular proteases of mycelial fungi as participants of pathogenic process. *Russian Journal of Bioorganic Chemistry* 34(3):286–289.
- Dunaevsky, Y. E., Elpidina, E. N., Vinokurov, K. S., & Belozersky, M. A. (2005). Protease inhibitors in improvement of plant resistance to pathogens and insects. *Molecular Biology* 39(4):608–613.
- Dutt, S., Gaur, V. S., Taj, G., & Kumar, A. (2012). Differential induction of two different cystatin genes during pathogenesis of Karnal bunt (*Tilletia indica*) in wheat under the influence of jasmonic acid. *Gene* 506(1):253–260.
- Eigner, D., & Scholz, D. (1999). *Ferula asafoetida* and *Curcuma longa* in traditional medical treatment and diet in Nepal. *Journal of Ethnopharmacology* 67(1):1–6.
- Ewing, B., Hillier, L. D., & Wendl, M. C. (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* 8(3):186–194.
- Expósito-Rodríguez, M., Borges, A. A., Borges-Pérez, A., & Pérez, J. A. (2008). Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biology* 8:131.
- Falco, M. C., & Silva-Filho, M. C. (2003). Expression of soybean proteinase inhibitors in transgenic sugarcane plants: effects on natural defense against *Diatraea saccharalis*. *Plant Physiology and Biochemistry* 41(8):761–766.
- Fang, E. F., Wong, J. H., & Ng, T. B. (2010). Thermostable Kunitz trypsin inhibitor with cytokine inducing, antitumor and HIV-1 reverse transcriptase inhibitory activities from Korean large black soybeans. *Journal of Bioscience and Bioengineering* 109(3):211–217.
- Farmer, E. E., Johnson, R. R., & Ryan, C. A. (1992). Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiology* 98(3):995–1002.

- Garcia-Seco, D., Zhang, Y., Gutierrez-Mañero, F. J., Martin, C., & Ramos-Solano, B. (2015). RNA-Seq analysis and transcriptome assembly for blackberry (*Rubus* sp. Var. Lochness) fruit. *BMC Genomics* 16:5.
- Gautam, A. K. (2014). *Colletotrichum gloeosporioides*: Biology, Pathogenicity and Management in India. *Journal of Plant Physiology Pathology* 2(2):1-11.
- Ghangal, R., Raghuvanshi, S., & Chand Sharma, P. (2009). Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites. *Plant Physiology and Biochemistry* 47(11-12):1113–1115.
- Goel, A., Kunnumakkara, A. B., & Aggarwal, B. B. (2008). Curcumin as “Curecumin”: from kitchen to clinic. *Biochemical Pharmacology* 75(4):787-809.
- Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., *et al.* (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* 29(7):644–652.
- Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., *et al.* (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols* 8(8):1494–1512.
- Habib, H., & Fazili, K. M. (2007). Plant protease inhibitors: A defense strategy in plants. *Biotechnology and Molecular Biology Review* 2(3):68–85.
- Haggag, H., & El-Gamal, G. (2012). In vitro study on *Fusarium solani* and *Rhizoctonia solani* isolates causing the damping off and root rot diseases in tomatoes. *Nature and Science* 10(11):16-25.
- Haq, S. K., Atif, S. M., & Khan, R. H. (2004). Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection. *Archives of Biochemistry and Biophysics* 431(1):145–159.
- Haruta, M., Major, I. T., Christopher, M. E., Patton, J. J., & Constabel, C. P. (2001). A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): Cloning, functional expression, and induction by wounding and herbivory. *Plant Molecular Biology* 46(3):347–359.
- Heath, M. C. (2000). Hypersensitive response-related death. *Plant Molecular Biology* 44(3):321–334.
- Hildmann, T., Ebner, M., Peña-Cortés, H., Sánchez-Serrano, J. J., Willmitzer, L., & Prat, S. (1992). General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *The Plant Cell* 4(9):1157–1170.
- Ho, C.-L., Tan, Y.-C., Yeoh, K.-A., Ghazali, A.-K., Yee, W.-Y., & Hoh, C.-C. (2016). De novo transcriptome analyses of host-fungal interactions in oil palm (*Elaeis guineensis* Jacq.). *BMC genomics*, 17(1):66.

- Höfte, M., & De Vos, P. (2007). Plant pathogenic *Pseudomonas* species. In *Plant-Associated Bacteria*, ed. S.S. Gnanamanickam, pp. 507-533. Dordrecht: Springer Netherlands.
- Honaas, L. A., Wafula, E. K., Wickett, N. J., Der, J. P., Zhang, Y., Edger, P. P., Altman, N. S., *et al.* (2016). Selecting superior de novo transcriptome assemblies: Lessons learned by leveraging the best plant genome. *PLoS ONE*, 11(1):1–42.
- Huang, H., Qi, S.-D., Qi, F., Wu, C.-A., Yang, G.-D., & Zheng, C.-C. (2010). NtKTI1, a Kunitz trypsin inhibitor with antifungal activity from *Nicotiana tabacum*, plays an important role in tobacco's defense response. *The FEBS Journal* 277(19):4076–4088.
- Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., Mueller, O., *et al.* (2005). Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Research* 33(6):1–12.
- Ingmer, H., & Brøndsted, L. (2009). Proteases in bacterial pathogenesis. *Research in Microbiology* 160(9):704–710.
- Jayaprakasha, G. K., Jagan Mohan Rao, L., & Sakariah, K. K. (2005). Chemistry and biological activities of *C. longa*. *Trends in Food Science & Technology* 16(12):533–548.
- Johnson, L. S., Eddy, S. R., & Portugaly, E. (2010). Hidden Markov model speed heuristic and iterative HMM search procedure. *BMC Bioinformatics*, 11, 431.
- Johnson, R., Narvaez, J., An, G., & Ryan, C. (1989). Expression of proteinase inhibitors I and II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences of the United States of America* 86(24):9871–9875.
- Johnson, S. L., & Pellecchia, M. (2006). Structure- and fragment-based approaches to protease inhibition. *Current topics in medicinal chemistry* 6(4):317–29.
- Jongsma, M. a., & Bolter, C. (1997). The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology* 43(10):P885–895.
- Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: A review of general principles and contemporary practices. *Clinical Infectious Diseases* 49(11):1749–1755.
- Kang, S.-G., Choi, J.-H., & Suh, S.-G. (2002). A leaf-specific 27 kDa protein of potato Kunitz-type proteinase inhibitor is induced in response to abscisic acid, ethylene, methyl jasmonate, and water deficit. *Molecules and Cells* 13(1):144–147.
- Kawasaki-Tanaka, A., & Fukuta, Y. (2014). Genetic variation in resistance to blast disease (*Pyricularia oryzae* Cavara) in Japanese rice (*Oryza sativa* L.), as determined using a differential system. *Breeding Science* 64(2):183–192.

- Kim, J. Y., Park, S. C., Hwang, I., Cheong, H., Nah, J. W., Hahm, K. S., & Park, Y. (2009). Protease inhibitors from plants with antimicrobial activity. *International Journal of Molecular Sciences* 10(6):2860–2872.
- Kim, J.-Y., Park, S.-C., Kim, M.-H., Lim, H.-T., Park, Y., & Hahm, K.-S. (2005). Antimicrobial activity studies on a trypsin-chymotrypsin protease inhibitor obtained from potato. *Biochemical and Biophysical Research Communications* 330(3):921–927.
- Koiwa, H., Bressan, R. A., & Hasegawa, P. M. (1997). Regulation of protease inhibitors. *Trends in Plant Science* 2(10):379-384.
- Krup, V., Prakash L, H., & A, H. (2013). Pharmacological Activities of Turmeric (*Curcuma longa* linn): A Review. *Journal of Homeopathy & Ayurvedic Medicine* 2(4).
- Kwok, S., Chang, S. Y., Sninsky, J. J., & Wang, A. (1994). A guide to the design and use of mismatched and degenerate primers. *Genome Research* 3(4):39–47.
- Langmead, B., Trapnell, C., Pop, M., Salzberg, S. L., Down, T., Rakyar, V., Turner, D., et al. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* 10(3):r25.
- Lantz, M. S. (1997). Are bacterial proteases important virulence factors? *Journal of Periodontal Research* 32(1 Pt 2):126–132.
- Lantz, R., Chen, G., Solyom, A., Jolad, S., & Timmermann, B. (2005). The effect of turmeric extracts on inflammatory mediator production. *Phytomedicine* 12(6-7):445–452.
- Lawrence, P. K., & Koundal, K. R. (2002). Plant protease inhibitors in control of phytophagous insects. *Electronic Journal of Biotechnology* 5(1):93-109.
- Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12(1):323.
- Li, H., Child, M. a, & Bogyo, M. (2012). Proteases as regulators of pathogenesis: examples from the Apicomplexa. *Biochimica et biophysica acta* 1824(1):177–185.
- Li, J., Brader, G., & Palva, E. T. (2008). Kunitz trypsin inhibitor: An antagonist of cell death triggered by phytopathogens and fumonisins B1 in *Arabidopsis*. *Molecular Plant* 1(3):482–495.
- Li, M., Su, Z. G., & Janson, J. C. (2004). In vitro protein refolding by chromatographic procedures. *Protein Expression and Purification* 33(1):1–10.
- Liener, I. E. (1970). Toxic constituents of plant foodstuffs. In *The Proceedings of the Nutrition Society* (Vol. 29). Academic Press.

- Lima, T. B., Silva, O. N., Migliolo, L., Souza-Filho, C. R., Goncalves, E. G., Vasconcelos, I. M., Oliveira, J. T. A., *et al.* (2011). A Kunitz proteinase inhibitor from corms of *Xanthosoma blandum* with bactericidal activity. *Journal of Natural Products* 74(5):969–975.
- Linhart, C., & Shamir, R. (2005). The degenerate primer design problem: Theory and applications. *Journal of Computational Biology* 12(4):431–456.
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Method* 25(4):402–408.
- Lomate, P. R., & Hivrale, V. K. (2012). Wound and methyl jasmonate induced pigeon pea defensive proteinase inhibitor has potency to inhibit insect digestive proteinases. *Plant Physiology and Biochemistry* 57:193–199.
- Lund, B. M., Baird-Parker, T. C., & Gould, G. W. (2000). *The microbiological safety and quality of food*. US:Springer-verlag.
- M. Polya, G. (2003). Protein and non-protein protease inhibitors from plants. *Studies in Natural Products Chemistry* 29(PART J):567–641.
- Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., *et al.* (1989). Mechanism of inhibition of papain by chicken egg white cystatin. Inhibition constants of N-terminally truncated forms and cyanogen bromide fragments of the inhibitor. *FEBS Letters* 243(2):234–238.
- Major, I. T., & Constabel, C. P. (2008). Functional analysis of the Kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. *Plant Physiology* 146(3):888–903.
- Mallory, P. A., & Travis, J. (1975). Inhibition spectra of the human pancreatic endopeptidases. *American Journal of Clinical Nutrition* 28(8):823–830.
- Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., *et al.* (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology* 13(6):614–629.
- Martinez, M., Abraham, Z., Gambardella, M., Echaide, M., Carbonero, P., & Diaz, I. (2005). The strawberry gene Cyf1 encodes a phytocystatin with antifungal properties. *Journal of Experimental Botany* 56(417):1821–1829.
- Martinez, M., Diaz-Mendoza, M., Carrillo, L., & Diaz, I. (2007). Carboxy terminal extended phytocystatins are bifunctional inhibitors of papain and legumain cysteine proteinases. *FEBS Letters* 581(16):2914–2918.
- Mccarthy, D. J., & Smyth, G. K. (2009). Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics* 25(6):765–771.
- Minoche, A. E., Dohm, J. C., & Himmelbauer, H. (2011). Evaluation of genomic high-throughput sequencing data generated on Illumina HiSeq and Genome Analyzer systems. *Genome Biology* 12(11):R112.

- Morton, R. L., Schroeder, H. E., Bateman, K. S., Chrispeels, M. J., Armstrong, E., & Higgins, T. J. (2000). Bean alpha-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. *Proceedings of the National Academy of Sciences of the United States of America* 97(8):3820–3825.
- Moura, D. S., & Ryan, C. a. (2001). Wound-inducible proteinase inhibitors in pepper. Differential regulation upon wounding, systemin, and methyl jasmonate. *Plant Physiology* 126(1):289–298.
- Mukherjee, S., Huntemann, M., Ivanova, N., Kyrpides, N. C., & Pati, A. (2015). Large-scale contamination of microbial isolate genomes by Illumina PhiX control. *Standards in Genomic Sciences* 10(1):18.
- Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R. J., Wigdahl, B., & Parveen, Z. (2008). Antiviral potentials of medicinal plants. *Virus research* 131(2):111–120.
- Newton, A. C., Fitt, B. D. L., Atkins, S. D., Walters, D. R., & Daniell, T. J. (2010). Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends in Microbiology* 18(8):365–373.
- Odjakova, M., & Hadjiivanova, C. (2001). The complexity of pathogen defense in plants. *Bulgarian Journal of Plant Physiology* 27:101–109.
- Okada, K., Wangpoengtrakul, C., Tanaka, T., Toyokuni, S., Uchida, K., & Osawa, T. (2001). Curcumin and especially tetrahydrocurcumin ameliorate oxidative stress-induced renal injury in mice. *The Journal of Nutrition* 131(8):2090–2095.
- Oliva, M. L. V, Silva, M. C. C., Sallai, R. C., Brito, M. V, & Sampaio, M. U. (2010). A novel subclassification for Kunitz proteinase inhibitors from leguminous seeds. *Biochimie* 92(11):1667–1673.
- Padmanabhan, S., Banerjee, S., & Mandi, N. (2011). Screening of bacterial recombinants: Strategies and preventing false positives. *Molecular Cloning - Selected Applications in Medicine and Biology* 3–20.
- Parlevliet, J. E. (2002). Durability of resistance against fungal, bacterial and viral pathogens; present situation. *Euphytica* 124(2):147–156.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in Real-Time RT-PCR. *Nucleic Acids Research* 29(9):e45.
- Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. *Biotechnology Letters* 26(6):509–515.
- Popovic, M. M., Bulajic, A., Ristic, D., Krstic, B., Jankov, R. M., & Gavrovic-Jankulovic, M. (2012). In vitro and in vivo antifungal properties of cysteine proteinase inhibitor from green kiwifruit. *Journal of the Science of Food and Agriculture* 92(15):3072–3078.

- Porruana, R., Chundetb, R., & Anuntalabhochaia, S. (2013). Characterization of a cDNA encoding cystatin with antifungal activity from Siam tulip *Curcuma alismatifolia*. *ScienceAsia* 39(6):596–604.
- Rawlings, N. D., & Barrett, A. J. (1999). MEROPS: The peptidase database. *Nucleic Acids Research* 27(1):325–331.
- Rawlings, N. D., Barrett, A. J., & Finn, R. (2015). Twenty years of the *MEROPS* database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Research* 44(D1):343–350.
- Reppond, K. C., & Babbitt, J. K. (1993). Protease inhibitors affect physical properties of arrowtooth flounder and walleye pollock surimi. *Journal of Food Science* 58(1):96–98.
- Richterich, P. (1998). Estimation of errors in “Raw” DNA sequences: A validation study. *Genome Research* 8(3):251–259.
- Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1):139–140.
- Rodrigues Macedo, M. L., Durigan, R. A., da Silva, D. S., Marangoni, S., Freire, M. das G. as M., & Parra, J. R. P. (2010). *Adenanthera pavonina* trypsin inhibitor retard growth of *Anagasta kuehniella* (Lepidoptera: Pyralidae). *Archives of Insect Biochemistry and Physiology* 73(4):213–231.
- Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*, 5(APR):1–17.
- Ryan, C. A. (1990). Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology* 28(1):425–449.
- San-Miguel, T., Perez-Bermudez, P., & Gavidia, I. (2013). Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. *Springerplus* 2(1):89.
- Sayle, R. A., & Milner-White, E. J. (1995). RASMOL: biomolecular graphics for all. *Trends in Biochemical Sciences* 20(9):374–376.
- Schaible, U. E., & Kaufmann, S. H. E. (2005). A nutritive view on the host-pathogen interplay. *Trends in Microbiology* 13(8):373–80.
- Scheel, D. (1998). Resistance response physiology and signal transduction. *Current Opinion in Plant Biology* 1(4):305–310.
- Schlüter, U., Benchabane, M., Munger, A., Kiggundu, A., Vorster, J., Goulet, M.-C., Cloutier, C., *et al.* (2010). Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective. *Journal of Experimental Botany*, 61(15):4169–4183.

- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols* 3(6):1101–1108.
- Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., *et al.* (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology* 7(1):3.
- Schulze-Lefert, P., & Panstruga, R. (2011). A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* 16(3):117–125.
- Scott, C. J., & Taggart, C. C. (2010). Biologic protease inhibitors as novel therapeutic agents. *Biochimie* 92(11):1681–1688.
- Seife, C. (1997). Blunting nature's swiss army knife. *Science* 277(5332):1602–1603.
- Sels, J., Mathys, J., De Coninck, B. M. a, Cammue, B. P. a, & De Bolle, M. F. C. (2008). Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiology and Biochemistry* 46(11):941–950.
- Shamsi, T. N., Parveen, R., & Fatima, S. (2016). Characterization, biomedical and agricultural applications of protease inhibitors: A review. *International Journal of Biological Macromolecules* 91:1120–1133.
- Shen, H.-B., & Chou, K.-C. (2009). Identification of proteases and their types. *Analytical Biochemistry* 385(1):153–160.
- Silver, N., Best, S., Jiang, J., & Thein, S. L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using Real-Time PCR. *BMC Molecular Biology* 7(1):33.
- Soares-Costa, A., Beltramini, L. M., Thiemann, O. H., & Henrique-Silva, F. (2002). A sugarcane cystatin: Recombinant expression, purification, and antifungal activity. *Biochemical and Biophysical Research Communications* 296(5):1194–1199.
- do Socorro M Cavalcanti, M., Oliva, M. L. V, Fritz, H., Jochum, M., Mentele, R., Sampaio, M., Coelho, L. C. B. B., *et al.* (2002). Characterization of a Kunitz trypsin inhibitor with one disulfide bridge purified from *Swartzia pickellii*. *Biochemical and Biophysical Research Communications* 291(3):635–639.
- Sookkongwaree, K., Geitmann, M., Roengsumran, S., Petsom, A., & Danielson, U. H. (2006). Inhibition of viral proteases by Zingiberaceae extracts and flavones isolated from *Kaempferia parviflora*. *Pharmazie*, 61(8):717–721.
- Sørensen, H. P., & Mortensen, K. K. (2005). Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microbial Cell Factories* 4(1):1.
- Srinivasan, A., Giri, A. P., Harsulkar, A. M., Gatehouse, J. a, & Gupta, V. S. (2005). A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts anti-metabolic effect on podborer (*Helicoverpa armigera*) larvae. *Plant Molecular Biology* 57(3):359–374.

- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28(10):2731–2739.
- Tan, Y. C., Wong, M. Y., & Ho, C. L. (2015). Expression profiles of defence related cDNAs in oil palm (*Elaeis guineensis* Jacq.) inoculated with mycorrhizae and *Trichoderma harzianum* Rifai T32. *Plant Physiology and Biochemistry* 96:296–300.
- Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2010). A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. *Methods* 50(4):S1–S5.
- The Wealth of India (2001). *An Encyclopedia of India's Raw Material Resources*. New Delhi: National Institute of Science Communication, CSIR.
- Travis, J., Potempa, J., & Maeda, H. (1995). Are bacterial proteinases pathogenic factors? *Trends in Microbiology* 3(10):405–407.
- Tsumoto, K., Ejima, D., Kumagai, I., & Arakawa, T. (2003). Practical considerations in refolding proteins from inclusion bodies. *Protein Expression and Purification* 28(1):1–8.
- Urwin, P. E., Atkinson, H. J., Waller, D. A., & McPherson, M. J. (1995). Engineered oryzacystatin-I expressed in transgenic hairy roots confers resistance to *Globodera pallida*. *The Plant Journal* 8(1):121–131.
- Valdes-Rodriguez, S., Cedro-Tanda, A., Aguilar-Hernandez, V., Cortes-Onofre, E., Blanco-Labra, A., & Guerrero-Rangel, A. (2010). Recombinant amaranth cystatin (AhCPI) inhibits the growth of phytopathogenic fungi. *Plant Physiology and Biochemistry* 48(6):469–475.
- Valueva, T. A., & Mosolov, V. V. (2004). Role of inhibitors of proteolytic enzymes in plant defense against phytopathogenic microorganisms. *Biochemistry* 69(11): 1305–1309.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7), RESEARCH0034.
- Vera, A., González-Montalbán, N., Arís, A., & Villaverde, A. (2007). The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. *Biotechnology and Bioengineering* 96(6):1101–1106.
- Vranova, V., Rejsek, K., & Formanek, P. (2013). Proteolytic activity in soil: A review. *Applied Soil Ecology* 70:23–32.

- Wang, H. X., & Ng, T. B. (2006). Concurrent isolation of a Kunitz-type trypsin inhibitor with antifungal activity and a novel lectin from *Pseudostellaria heterophylla* roots. *Biochemical and Biophysical Research Communications* 342(1):349–353.
- Wang, H.-Y., Huang, Y.-C., Chen, S.-F., & Yeh, K.-W. (2003). Molecular cloning, characterization and gene expression of a water deficiency and chilling induced proteinase inhibitor I gene family from sweet potato (*Ipomoea batatas* Lam.) leaves. *Plant Science* 165(1):191–203.
- Wang, K. M., Kumar, S., Cheng, Y. S., Venkatagiri, S., Yang, A. H., & Yeh, K. W. (2008). Characterization of inhibitory mechanism and antifungal activity between group-1 and group-2 phytocystatins from taro (*Colocasia esculenta*). *FEBS Journal* 275(20):4980–4989.
- Wang, L., & Stegemann, J. P. (2010). Extraction of high quality RNA from polysaccharide matrices using cetyltrimethylammonium bromide. *Biomaterials* 31(7):1612–1618.
- Williamson, M. S., Forde, J., Buxton, B., & Kreis, M. (1987). Nucleotide sequence of barley chymotrypsin inhibitor-2 (CI-2) and its expression in normal and high-lysine barley. *The Federation of European Biochemical Societies Journal* 165(1):99–106.
- van Wyk, S. G., Kunert, K. J., Cullis, C. A., Pillay, P., Makgopa, M. E., Schlüter, U., & Vorster, B. J. (2016). Review: The future of cystatin engineering. *Plant Science* 246:119–127.
- Xie, F., Xiao, P., Chen, D., Xu, L., & Zhang, B. (2012). miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molecular Biology* 80(1):75–84.
- Zhang, H., Xie, X., Xu, Y., & Wu, N. (2004). Isolation and functional assessment of a tomato proteinase inhibitor II gene. *Plant Physiology and Biochemistry*, 42(5):437–444.
- Zhang, S., An, S., Li, Z., Wu, F., Yang, Q., Liu, Y., Cao, J., *et al.* (2015). Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in *Helicoverpa armigera* (lepidoptera: Noctuidae). *Gene* 555(2):393–402.
- Zhang, Y., Lubberstedt, T., & Xu, M. (2013). The genetic and molecular basis of plant resistance to pathogens. *Journal of Genetics and Genomics* 40(1):23–35.