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

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

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

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

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

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

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

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

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	mililitre
mg	miligram
M	molar
w/v	weight per volume
v/v	volume per volume
µL	microlitre
mg	miligram
g	gram
mM	milimolar
V	volt
bp	base pair
ng	nanogram
OD	optical density
pI	isoelectric point
N50	median of lengths in NGS genome assembly

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 occurence 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].

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., & Ibnsouda, 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 A 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 col*i. *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., Ebneth, 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., Rakyan, 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 fumonisin 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 cetlytrimethylammonium 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.