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Engineering plant cysteine protease inhibitors for the transgenic control of banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) and other coleopteran insects in transgenic plants

By

ANDREW KIGGUNDU

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Department of Plant Sciences and Forestry and Agricultural Biotechnology Institute (FABI)
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University of Pretoria South Africa

Supervisor:
PROF. K.J. KUNERT

Co-supervisors:
PROF. A. VILJOEN
PROF. D. MICHAUD

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TABLE OF CONTENTS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF FIGURES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

LIST OF TABLES

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Andrew Kiggundu
National Banana Research Programme, Kawanda Agricultural Research Institute,
National Agricultural Research Organisation,
P. O. Box 7065, Kampala, Uganda

Department of Plant Science and the Forestry and Agricultural Biotechnology Institute
University of Pretoria, 74 Lunnon Road, Hillcrest,
Pretoria, 0002. South Africa

Supervisor:

Karl Kunert
Department of Plant Science and the Forestry and Agricultural Biotechnology Institute
University of Pretoria, 74 Lunnon Road,
Hillcrest, Pretoria, 0002. South Africa

Co-supervisors:

Altus Viljoen
Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute University of Pretoria, 74 Lunnon Road,
Hillcrest, Pretoria, 0002. South Africa

Currently at: Department of Plant Pathology, University of Stellenboch, Private Bag X1, Matieland 7602, South Africa.

Dominique Michaud
Départment de Phytologie, Pavillon Paul-Comtois,
Université Laval, Sainte-Foy (Québec),
Canada G1K 7P4

ABSTRACT

Cysteine protease inhibitors (cystatins) are expressed in plants in response to wounding and insect herbivory and they form part of the native host-plant defence system. Cysteine proteases are enzymes important in the break down of dietary proteins mainly in the mid gut of coleopteran insects such as the banana weevil. The inhibition of these proteases has a direct effect on the digestive activity of the insect resulting in protein deficiency. This significantly affects insect development and survival. Based on these observations, strategies have been designed involving expression of cysteine protease inhibitors for the transgenic control of insect pests of several crop plants. For this study, it was hypothesized that the major proteases in banana weevil are cysteine proteases and can be effectively targeted by plant cystatins. It was further hypothesised that since plant cystatins are defense related, certain amino acid residues may have undergone positive selection. This provides an opportunity to increase their inhibitory potential to the weevil gut proteases via protein engineering. To prove the hypotheses, both *in-vitro* and *in-vivo* assays were set up thus allowing us to demonstrate the presence of cysteine type proteases banana weevil as well as the effect of cystatins on the weevil proteases and early development. Initial *in-vitro* experiments were able to characterize the proteolytic activity of the banana weevil gut proteases, which are mostly of the cysteine type, and in particular cathepsin B and L like. Two recombinant phytocystatins were further successfully produced using a 6xHis-tagged affinity chromatogephy system in *Escherichia coli* bacteria. The recombinant phytocystatins were used in a newly developed vacuum infiltration assay system using banana stems. Young weevil larvae were allowed to develop on phytocystatin-treated stems for up to 10 days. They had a 60% reduction in body weight and rate of growth compared to those that grew in untreated stems. By carrying out site-directed mutagenesis to improve the inhibition efficiency of a model papaya cystatin, more than 8 amino acid residues were found to be subjected to positive selection. Mutation of amino acids yielded improved the inhibition potential of papaya cystatin against the model cysteine protease papain. Increased inhibition was greatest when amino acids were changed in the highly variable regions of the amino acid sequence very closely to the conserved regions.

This study has been able to show for the first time that banana weevils use cysteine protease as major protein hydrolysis enzymes and that these can be effectively targeted by plant cystatins. It has also created novel phytocystatins using engineering of single amino acid sites following an evolutionary approach to modulate them for improved activity and targeting specific proteases.

THESIS COMPOSITION

Chapter 1 introduces the banana weevil which is a coleopteran pest of banana that barrows through the underground stem of banana plants causing considerable damage. The chapter reviews conventional efforts towards screening the banana germplasm for resistance, resistance mechanisms, and cross breeding activities targeting the banana weevil as well as protease inhibitors as one group of genes that have potential for weevil control in a transgenic approach. **Chapter 2** reports on investigations into the nature of the banana weevil gut environment *vis* a *vis* protease activity reveals the protease profile of the gut and bioassays are developed and conducted to test the hypothesis that banana weevil use mostly cysteine protease in protein digestion and can be targeted by cysteine protease inhibitors from plants. **Chapter 3** relates to the phlylogeneic, structural and protein modelling analysis of plant cysteine protease inhibitors in an effort to understand evolutionary trends. This could assist a protein engineering strategy to improve the cystatin action against weevil and other coleopteran insects. **Chapter 4** combines evolutionary analysis to determine if positive selection has acted on the cysteine protease inhibitor amino acid residues to lead to the observed diversity. This was followed by protein engineering approaches using site-directed mutagenesis guided by evolutionary analysis to produce novel mutants of the papaya cystatin with increased inhibition capacity. Finally **Chapter 5** discusses the contributions of this thesis to our better understanding of these important plant proteins. It further discusses how best to make future use of them, not only in the improvement of resistance to banana weevil but also to other coleopteran crop pests.

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ABBREVIATIONS AND SYMBOLS

BBTI	Bowman-Birk trypsin inhibitor
bp	Base pair
CaMV	Cauliflower Mozaic Virus
E-64	Trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane
EDTA	Ethylenediaminetetraacetic acid
kDa	Killo Dalton
LB	Luria-Bertani
mL	Milliliter
nm	Nanometer
OC-I	oryzacystatin-I
PAGE	Polyacrylamide gel electrophoresis
PC	Papaya cystatin
PCR	Polymerase Chain Reaction
PI	Protease inhibitor
PMSF	Phenylmethylsulphonyl fluoride
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
U	Unit
Z-phe-arg-AMC	Benzyloxycarbonyl-phenylalanine-arginie aminomythylcoumarin
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
°C	Degree Celsius
m	Metre