Gene expression and plant performance in oryzacystatin-I expressing transformed tobacco (Nicotiana tabacum L. cv Samsun) plants under abiotic stress.

By

GETU BEYENE

Thesis submitted in partial fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR

Forestry and Agricultural Biotechnology Institute (FABI)

Department of Botany

in the

Faculty of Natural and Agricultural Sciences

University of Pretoria

SUPERVISORS: PROF. K.J. KUNERT

PROF. C.H. FOYER

March 2006
CONTENTS                                                                 PAGES
ABSTRACT .......................................................................................................................... viii
RESEARCH AIMS AND OBJECTIVES ............................................................................. x
THESIS COMPOSITION ..................................................................................................... xi
ACKNOWLEDGEMENTS ................................................................................................xiii
ABBREVIATIONS AND SYMBOLS ................................................................................. xv
LIST OF FIGURES ............................................................................................................ xvii
LIST OF TABLES ................................................................................................................ xx
CHAPTER ONE ..................................................................................................................... 1
  1.1 Plants and stress ...................................................................................................... 2
    1.1.1 Forms of plant stress ........................................................................................ 2
    1.1.2 Drought and heat stress in plants .................................................................... 3
      1.1.2.1 Drought stress ......................................................................................... 3
      1.1.2.2 Heat stress ................................................................................................ 6
  1.2 Plant gene expression under stress ........................................................................ 7
    1.2.1 Techniques to detect gene expression .............................................................. 7
    1.2.2 Gene expression under drought stress ............................................................ 8
    1.2.3 Gene expression under heat stress ................................................................. 14
  1.3 Plant engineering for drought and heat stress tolerance ........................................ 15
    1.3.1 Plant engineering for drought stress .............................................................. 15
    1.3.2 Plant engineering for heat stress ................................................................. 19
  1.4 Proteinase/proteinase inhibitor system and stress ................................................. 21
    1.4.1 Plant proteinases .......................................................................................... 21
      1.4.1.1 Cysteine proteinases ............................................................................. 21
1.4.1.2 Cysteine proteinase expression under drought and heat stress .......... 28
1.4.2 Plant proteinase inhibitors................................................................. 35
1.4.2.1 Cysteine proteinase inhibitors...................................................... 36
1.4.2.2 Plant proteinase inhibitor expression under drought and heat stress .... 39
1.4.3 Plant engineering and the cysteine proteinase/proteinase inhibitor system.... 42
1.4.3 Stability of proteinase inhibitors transgenes in plants ....................... 46

CHAPTER TWO .................................................................................................................. 50

EXPRESSION OF ORYZACYSTATIN-I IN DROUGHT AND HEAT-STRESSED
TRANSFORMED TOBACCO PLANTS ........................................................................... 50

2.1 Abstract ........................................................................................................... 51

2.2 Introduction ..................................................................................................... 52

2.3 Materials and Methods .................................................................................. 54

2.3.1 Plant material ............................................................................................. 54
2.3.2 Detection of OC-I sequence in transgenic plants ....................................... 54
2.3.3 Stress treatment of plants .......................................................................... 55
2.3.4 Preparation of leaf protein extract .............................................................. 56
2.3.5 Immuno-blotting ........................................................................................ 56
2.3.6 Cysteine proteinase inhibition by tobacco leaf protein extract .............. 57
2.3.7 Southern blot analysis .............................................................................. 58
2.3.8 Northern blot analysis ............................................................................ 58

2.4 Results ............................................................................................................. 60

2.4.1 OC-I gene and protein detection .............................................................. 60
2.4.2 Expression of the small and large subunit of Rubisco ............................ 62
2.4.3 OC-I expression under stress ................................................................. 64
2.4.4 Activity of expressed OC-I ................................................................. 66

2.5 Discussion ........................................................................................................ 68

CHAPTER THREE ............................................................................................................... 70

GROWTH OF OC-I EXPRESSING TRANSFORMED TOBACCO PLANTS UNDER
ABIOTIC STRESS ............................................................................................................... 70

3.1 Abstract ................................................................................................................. 71

3.2 Introduction ........................................................................................................... 72

3.3 Materials and Methods ......................................................................................... 74

3.3.1 Glasshouse experiments .................................................................................. 74

3.3.2 Growth cabinet experiments ........................................................................... 75

3.3.3 Statistical analysis ........................................................................................... 76

3.4 Results .................................................................................................................... 77

3.4.1 Effect of drought stress on plant growth and net photosynthetic rate in the
glasshouse ....................................................................................................................... 77

3.4.2 Effect of drought and heat stresses on plant growth and net photosynthetic rate
(growth cabinet) .............................................................................................................. 79

3.5 Discussion ............................................................................................................... 82

CHAPTER FOUR ................................................................................................................. 85

APPLICATION OF cDNA REPRESENTATIONAL DIFFERENCE ANALYSIS
(cDNA RDA) FOR DETECTION OF DIFFERENTIALLY EXPRESSED GENES IN
OC-I EXPRESSING TOBACCO ........................................................................................ 85

4.1 Abstract ..................................................................................................................... 86
4.2 Introduction ........................................................................................................... 87
4.3 Materials and Methods .......................................................................................... 89
  4.3.1 mRNA isolation and cDNA synthesis ............................................................ 89
  4.3.2 cDNA RDA ..................................................................................................... 89
    4.3.2.1 Amplicon production ................................................................................ 90
    4.3.2.2 First round subtraction and amplification .............................................. 92
    4.3.2.3 Second round subtraction and amplification .......................................... 94
    4.3.2.4 Third and fourth round subtraction and amplification ......................... 94
    4.3.2.5 Cloning and sequence analysis of difference products ....................... 95
  4.3.4 Gene expression under heat stress ................................................................ 95
  4.3.5 Chlorophyll determination ............................................................................ 96
  4.3.6 Two-dimensional gel electrophoresis (2-DE) ............................................... 96
4.4 Results ................................................................................................................. 99
  4.4.1 cDNA RDA ..................................................................................................... 99
  4.4.2 Expression of GBDP4-5d-12 ....................................................................... 104
  4.4.3 Chlorophyll content ...................................................................................... 105
  4.4.4 Protein expression detected by 2-DE ......................................................... 107
4.5 Discussion ............................................................................................................ 109

CHAPTER FIVE ................................................................................................................ 113

CLONING OF TWO NEW CYSTEINE PROTEINASES WITH SPECIFIC
EXPRESSION PATTERNS IN MATURE AND SENESCENT TOBACCO LEAVES
........................................................................................................................................ 113

5. 1 Abstract .............................................................................................................. 114
SUMMARY AND PERSPECTIVE.......................................................................................... 147
REFERENCES.................................................................................................................... 151

Getu Beyene
Department of Botany, Forestry and Agricultural Biotechnology Institute, University of Pretoria, 74 Lunnon Road, Hillcrest 0002, Pretoria, South Africa.

Supervisor: Professor Karl J. Kunert
Department of Botany, Forestry and Agricultural Biotechnology Institute, University of Pretoria, 74 Lumnnon Road, Hillcrest 0002, Pretoria, South Africa.

Co-supervisor: Professor Christine H. Foyer
Crop Improvement Division, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK.

ABSTRACT

Plant cysteine proteinase inhibitors or also called phytocystatins inhibit the action of cysteine proteinases in plants. These proteinases are involved in many developmental processes by degrading proteins. In this study possible effects of an exogenous oryzacystatin-I (OC-I) expressed in transformed tobacco has been investigated. By challenging OC-I expressing and non-expressing tobacco with drought and heat stress, OC-I transcription and translation were not affected in OC-I expressing plants and plant extracts from stressed plants containing the inhibitor inhibited papain activity *in vitro*. Further, plant growth and photosynthesis was not greatly different under the selected growth conditions in both plant types under stress and non-stress conditions. However, OC-I expressing plants showed slightly lower photosynthetic rate, were shorter and had a higher lower dry mass production under non-stress condition. By applying cDNA Representational Difference Analysis (cDNA-RDA) to detect differentially expressed genes in the two types of plants, a gene coding for the light harvesting chlorophyll *a/b* binding protein gene (*lhcb1*) of photosystem II (LHC II) was
isolated from non-OCI expressing plants. Northern blot analysis showed lower transcript accumulation of the *lhcb1* gene in OCI-expressing plants both under non-stress and stress conditions, which was accompanied by lower chlorophyll content in OC-I expressing plants. Furthermore, plants benefited from OC-I expression by protection of a variety of expressed proteins against degradation. Identification of possible target cysteine proteinases for OC-I in tobacco resulted in the isolation, cloning and characterization of two new papain-like cysteine proteinases from tobacco designated *NtCP1* and *NtCP2*. *NtCP1* was expressed only in senescent leaves and it was not induced in mature green leaves upon exposure to drought or heat stress. *NtCP1* has therefore a possible potential as a developmental senescence marker in tobacco. In contrast, *NtCP2*, which was expressed in mature green leaves, has a high similarity to KDEL-tailed cysteine proteinases that are involved in programmed cell death. Both drought and heat decreased *NtCP2* transcript abundance in mature green leaves. Overall, this study has provided evidence that expression of exogenous OC-I does not significantly improve plant performance in tobacco in terms of physiological traits under drought and heat stress but provides protection in terms of stability of protein expression by possibly interacting with endogenous tobacco cysteine proteinases. Further detailed studies are suggested on the interaction of endogenous cysteine proteinases and exogenous phytocystatins to elucidate in more detail the type of interaction.
RESEARCH AIM AND OBJECTIVES

Genetic engineering of plants, which involves the transfer of a single or multiple genes of interest to a plant genome, have been widely used both for introduction of desirable traits to plants and for a basic molecular biology study of gene function. A significant number of plants that have been transformed with stress tolerance genes have been generated. Evidences, however, suggest that the introduction of such genes into plant genome may not always result in desirable abiotic stress tolerant phenotype. This can partially be attributed to the level of expression of the transgene as well as subsequent stability of the transgene encoded protein under abiotic stress. Undesirable interaction of the introduced transgene with plant normal function has been also a frequent phenomenon. In this PhD study, it was hypothesized that constitutive overexpression of a rice cysteine proteinase inhibitor transgene (OC-I) in tobacco could confer protection against abiotic stresses, such as drought and heat. The aim of this study was to compare OC-I expressing tobacco plants with non-transformed plants both at physiological and molecular level in order to prove the working hypothesis that OC-I could confer protection against abiotic stresses. The specific objectives were to: (1) study the expression and stability of the OC-I transgene under drought and heat stress, (2) evaluate growth performance of transformed and non-transformed plants under drought and heat stress, (3) isolate differentially expressed genes between transformed and non-transformed plants under heat stress by using a technique of representational difference analysis of cDNA (cDNA-RDA) and (4) clone tobacco cysteine proteinases that could be possible endogenous targets of exogenous OC-I.
Chapter one reviews the current knowledge about plant responses to drought and heat stress. This chapter in particular covers the present knowledge on genes that have been identified and investigated to respond to drought and heat and have also been used to enhance stress tolerance. Further, this chapter provides in greater detail an overview about previous and current research on the different types of plant proteinases and proteinase inhibitors, their action and location in plants and their involvement in plant stress reactions. Chapter two reports on the characterization of transformed tobacco, which expresses an exogenous rice cysteine proteinase inhibitor (OC-I) gene. In particular, the chapter deals with detection of inhibitor integration into the plant genome and expression of the inhibitor in transformed tobacco under drought and heat stress. Chapter three compares, by measuring a variety of physiological parameters, plant performance of OC-I expressing and non-expressing tobacco plants under drought and heat stress and combination of both stresses to evaluate any benefit for plants of exogenous OC-I expression. This chapter reports about studies that have been carried out in the greenhouse and in environmentally controlled growth chambers. Chapter four presents results of the isolation of gene sequences differentially expressed between OC-I expressing plants and non-expressing plants in response to heat treatment by applying the technique of c-DNA Representational Difference Analysis (cDNA-RDA). In particular, results of expression of a sequence coding for a chlorophyll-binding protein under heat stress are reported. Finally, this chapter also deals with results obtained for pigment production and protein expression patterns in OC-I expressing and non-expressing tobacco under stress and non-stress conditions using spectro-photometry for pigment content determination and two-
dimensional gel electrophoresis (2DE) for detection of expressed proteins. Chapter five describes the cloning and detailed characterization of two new papain-like cysteine proteinases from tobacco leaves. This chapter also presents the expression patterns of these proteinases in response to drought, heat and combination of both stresses. Chapter six summarizes the new aspects of the study. This chapter specifically focuses on how the study has contributed to an advanced understanding of the consequences of exogenous OC-I expression in tobacco and in particular the benefits gained from OC-I expression but also its limitation. Finally, this chapter also outlines possible future research activities including the isolation and characterization of endogenous cysteine proteinases that might interact with expressed exogenous inhibitors.
ACKNOWLEDGEMENTS

I could have taken a wrong detour than being in academic environment had it not been for the caring father and mother. I thank both for bringing me up to the level where I am today.

My sincere thanks to my supervisor, Prof Karl Kunert for all his patience, support and guidance through out this study. Two moments were very special, first dating back to September 2001 for facilitating my transfer from the Kasetsart University in Bangkok to the University of Pretoria and second the opportunities he gave me to visit my family whom I missed most during this study. I would also thank my co-supervisor Prof. Christeine Foyer for giving me the privillage to visit her lab at Rothamsted Research and learn proteomics.

I am very much indepted to Alemaya University for sponsoring my study. Special thanks to Prof. Belay Kassa for all the support he rendered to me and my family. My sincere gratitude also to colleagues and friends at Forestry and Agricultural Biotechnology Institute (FABI), the University of Pretoria, who suppoted me during my study.

It also gives me a pleasure to thank best friends and colleagues, Yoseph Beyene, Tekalign Tsegaw, Abubakar Hassen, Solomon Kebede and Geremew Eticha and Mesfin Bogale for all their support during this study and advises in life. Tesfaye Lemma, Eyassu Seifu, Solomon Worku, Wondemeneh A., Yared M. and Melaku Z. are also acknowledged.
Last but not least I would like to thank my wife Mulualem Geleta and my Son Kena Getu for their patience and support during my absences for more than three years. I am also pleased to welcome our second boy who came to this world on the 7th of this month while this thesis was getting in shape.
**ABBREVIATIONS AND SYMBOLS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ABRE</td>
<td>ABA-responsive element</td>
</tr>
<tr>
<td>APX</td>
<td>Ascorbate peroxidase</td>
</tr>
<tr>
<td>AREB</td>
<td>ABRE-binding proteins</td>
</tr>
<tr>
<td>BBTI</td>
<td>Bowman-Birk trypsin inhibitor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbondioxide</td>
</tr>
<tr>
<td>COR15A</td>
<td>Cold-Regulated 15A</td>
</tr>
<tr>
<td>DRE/CRT</td>
<td>Dehydration-responsive element/C-repeat</td>
</tr>
<tr>
<td>DREB1/CBF</td>
<td>DRE/CRT binding protein</td>
</tr>
<tr>
<td>E-64</td>
<td>Trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESTs</td>
<td>Expressed Sequence Tag sequencing</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive</td>
</tr>
<tr>
<td>HSF</td>
<td>Heat shock factors</td>
</tr>
<tr>
<td>HSPs</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>IPM</td>
<td>Integrated pest management</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>Killo Dalton</td>
</tr>
<tr>
<td>KIN</td>
<td>Cold-inducible</td>
</tr>
<tr>
<td>LEA</td>
<td>Late embryogenesis abundant proteins</td>
</tr>
<tr>
<td>LHC II</td>
<td>light harvesting chlorophyll a/b binding protein of photosystem II</td>
</tr>
<tr>
<td>LSU</td>
<td>Large subunit</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>MeJA</td>
<td>Methyl jasmonate,</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MPSS</td>
<td>Massively Parallel Signature Sequencing</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OC-I</td>
<td>Oryzacystatin-I</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PhyCys</td>
<td>Phytocystatin</td>
</tr>
<tr>
<td>PI</td>
<td>Proteinase inhibitor</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>rbcL</td>
<td>Gene coding for large subunits of Rubisco</td>
</tr>
</tbody>
</table>
$rbcS$  Gene coding for small subunits of Rubisco
rd29A  Responsive to dehydration rd29A
RDA  Representational Difference Analysis
ROS  Reactive oxygen species
Rubisco  Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC,  Relative water content
SAG  Senescence associated gene
SAGE  Serial Analysis of Gene Expression
SD  Standard deviation
SDG  Senescence down-regulated gene
SDS  Sodium dodecyl sulphate
SE  Standard error
SO$_2$  Sulfur dioxide
SSU  Small subunit
TBS  Tris-buffered saline
TBS-T  Tris-buffered saline-Tween
TMV  Tobacco mosaic virus-1
U  Unit
UTR  Untranslated regions
UV  Ultra violet
VPE  Vacuolar processing enzyme
Z-phe-arg-AMC  Benzyloxycarbonyl-phenylalanine-arginie aminomethylcoumarin
$\alpha$-AI-1  $\alpha$-amylase inhibitor 1
$\mu$g  microgram
$\mu$l  Microlitre
$\mu$M  Micromolar
%  Percentage
°C  Degree Celsius
2DE  Two-dimensional gel electrophoresis
m  Metre
LIST OF FIGURES

Figure 1.1 Biotic and abiotic stresses that affect plant growth and development......................... 3

Figure 1.2 Techniques for gene expression profiling technologies............................................. 8

Figure 1.3 Regulatory networks of cis-acting elements and transcription factors involved in osmotic- and cold-stress responsive gene expression..................................................... 10

Figure 1.4 Drought stress inducible genes and their possible functions in stress tolerance and response................................................................................................................... 13

Figure 1.5 Genes that have been used to enhance abiotic stress tolerance in plants.............. 17

Figure 1.6 Functions of plant cysteine proteinases................................................................. 22

Figure 2.1 Characterization of putative transformed plants..................................................... 61

Figure 2.2 (A) Immuno-blot analysis of the a large subunit (LSU) and small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in transformed (T) and non-transformed (NT) tobacco plants under non-stress (C), drought (D), heat (H) and combined drought and heat stress (D&H)................................................. 63

Figure 2.3 (A) Immuno-blot analysis to detect expression of OC-I in transformed non-stressed tobacco (C) and in transformed tobacco plants exposed to drought (D), heat (H) and a combination of drought and heat stress (D&H)................................. 65

Figure 2.4 Residual papain activity (%) in the presence of 100 μg of plant soluble protein. 67

Figure 3.1 Growth characteristics of non-stressed, transformed (T) and non-stressed, non-transformed (NT) tobacco plants and drought-stressed transformed (TS) and drought-stressed non-transformed (NTS) tobacco plants........................................... 78
Figure 3.2 Plant growth and net photosynthetic rates of non-transformed and transformed plants under drought (D), heat (H) or a combination of both stresses (D&H). .... 81

Figure 4.1 (A) Amplicons of heat-stressed transformed (T) and non-transformed (NT) plant for cDNA RDA.............................................................. 101

Figure 4.2 Alignment of deduced amino acid sequences. (A) GBDP4-5d-12 amino acid sequence alignment with the amino acid sequence of the plant light harvesting chlorophyll a/b binding protein derived from different plant species. (B) GBDP4-3d-7 amino acid sequence alignment with the L12 ribosomal protein amino acid sequences derived from different plant species. .................................................. 103

Figure 4.3 Temporal expression of a GBDP4-5d-12 under non-stress and during heat stress exposure. Total RNA was isolated from transformed and non-transformed tobacco plants that have been either non-stressed (C, control) or heat-stressed for various time periods.......................................................... 105

Figure 4.4 (A) Chlorophyll a and (B) chlorophyll b content (mg g⁻¹ on fresh weight basis) of transformed and non-transformed tobacco leaves under non-stress and after heat stress exposure.......................................................... 106

Figure 4.5 Two-dimensional gel electrophoresis of a plant leaf protein extract derived from an OC-I expressing (transformed) and a non-transformed tobacco plant........ 108

Figure 5.1 (A) Tobacco plant and (B) developmental stages of leaves with progression of natural senescence. For the study fully expanded mature green (G3) and senescent
leaves (S7) were used. (C) Soluble protein and chlorophyll content of mature green (G3) and senescent (S7) leaf material. .......................................................... 128

**Figure 5.2** (A) Total proteinase activity in non-senescent, green (G) and senescent (S) tobacco leaves determined by activity gel electrophoresis on a mildly denaturing SDS-PAGE containing 0.1% gelatine and detection of proteinase activity after incubation with or without (Control) proteinases inhibitors.......................... 129

**Figure 5.3** Nucleotide and deduced amino acid sequence of NtCP1 (A) and NtCP2 (B).... 133

**Figure 5.4** Multiple alignment of NtCP1 with related senescence-associated proteinases (A) and NtCP2 with related KDEL-tailed proteinases (B)....................... 137

**Figure 5.5** Phylogenetic tree of plant papain-like cysteine proteinases....................... 139

**Figure 5.6** Southern blot analysis of NtCP1 and NtCP2............................................. 140

**Figure 5.7** (A) Northern blot analysis for detection of NtCP1 and NtCP2 expression in mature green and senescent leaves... .......................................................... 142
LIST OF TABLES

Table 1.1 Mechanisms, genes, genetically modified plants and enhanced tolerance to abiotic stress (adapted from Wang et al., 2003). ................................................................. 18

Table 1.2 Proteinases and PIs induced/repressed under different stresses and treatments .... 33

Table 1.3 Families of plant proteinaceous proteinase inhibitors........................................ 36

Table 1.4 Transgenic plants expressing cystatin genes for defense against pests and physiological studies (adapted from Haq et al., 2004). ............................................. 46

Table 3.1 Effect of drought stress on photosynthesis, stomatal conductance and transpiration rates of non-transformed and transformed tobacco plants (data are mean ± S.E. of 10 individual plants). .............................................................. 79

Table 4.1 Oligonucleotide adapters and primers used for cDNA RDA ......................... 91