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

By

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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 *Nt*CP1 and *Nt*CP2. *Nt*CP1 was expressed only in senescent leaves and it was not induced in mature green leaves upon exposure to drought or heat stress. *Nt*CP1 has therefore a possible potential as a developmental senescence marker in tobacco. In contrast, *Nt*CP2, 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 *Nt*CP2 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 enginnering of plants, which involves the transfer of a sigle 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 siginificant 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 results 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 nomal 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 confere 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-trasformed plants under drought and heat stress, (3) isolate differentially expressed genes between transformed and non-trasformed 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 endogeous targets of exogenous OC-I.

THESIS COMPOSITION

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.

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ABBREVIATIONS AND SYMBOLS

ABA	Abscisic acid
ABRE	ABA-responsive element
APX	Ascorbate peroxidase
AREB	ABRE-binding proteins
BBTI	Bowman-Birk trypsine inhibitor
bp	Base pair
CaMV	Cauliflower Mosaic Virus
CO_2	Carbondioxide
COR15A	Cold-Regulated 15A
DRE/CRT	Dehydration-responsive element/C-repeat
DREB1/CBF	DRE/CRT binding protein
E-64	T*rans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ESTs	Expressed Sequence Tag sequencing"
g	Gram
HR	Hypersensitive
HSF	heat shock factors
HSPs	Heat shock proteins
IPG	Immobilized pH gradient
IPM	Integrated pest management
JA.	Jasmonic acid
kDa	Killo Dalton
KIN	Cold-inducible
LEA	Late embryogenesis abundant proteins
LHC II	light harvesting chlorophyll a/b binding protein of photosystem II
LSU	Large subunit
M	Molarity
MeJA	Methyl jasmonate,
mL	Milliliter
MPSS	Massively Parallel Signature Sequencing
nm	Nanometer
OC-I	Oryzacystatin-I
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PhyCys	Phytocystatin
PI	Proteinase inhibitor
PMSF	Phenylmethylsulphonyl fluoride
RACE	Rapid amplification of cDNA ends
rbcL	Gene coding for large subunits of Rubisco

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-i
U	Unit
UTR	Untranslated regions
UV	Ultra violet
VPE	Vacuolar processing enzyme
Z-phe-arg-AMC	Benzyloxycarbonyl-phenylalanine-arginie aminomythylcoumarin
α -AI-1	α -amylase inhibitor 1
µg	microgram
µl	Microlitre
µM	Micromolar
%	Percentage
°C	Degree Celsius
2DE	Two-dimensional gel electrophoresis
m	Metre

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Plants and stress

1.1.1 Forms of plant stress

Plants being immobile, unlike animals, encounter challenges from environmental stresses to which they can adapt by altering morphological, physiological and developmental processes. Lichtenthaler (1998) defines the term stress as any unfavourable condition or substance that affects or blocks a plant's metabolism, growth or development, which can be induced by various natural and anthropogenic factors. The duration and severity stress determines the kind of plant response (Lichtenthaler, 1998). Consequently, understanding the way stress affects plants and the processes underlying plant responses to stress leading to tolerance/avoidance mechanisms will enable the improvement of plants through breeding strategies.

Stress factors are divided into biotic (living) and abiotic (non-living) stresses. Whereas biotic stresses include a variety of pathogenic microrganisms and higher animals including interferences from humans, abiotic stresses include water logging, drought, and extremes of temperature, wind, storm, lightening, intense light, excessive soil salinity, inadequate or excess mineral nutrients and also treatment with plant growth regulators and antibiotics (Figure 1.1). Among the environmental abiotic stresses, water deficit limits global food productivity more severely than any other environmental factor (Boyer, 1982; Araus *et al*., 2002) and drought is the major abiotic stress in many parts of the world (Johansen *et al*., 1992).

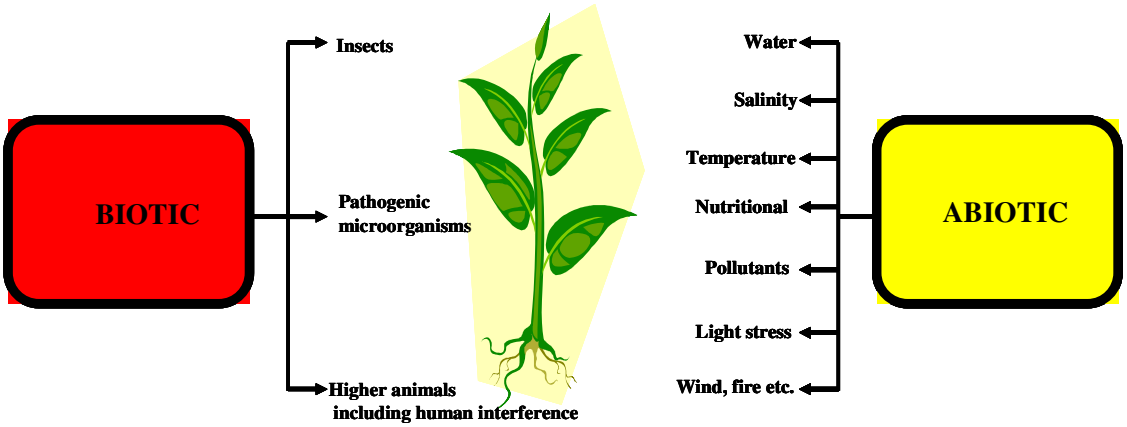


Figure 1.1 Biotic and abiotic stresses that affect plant growth and development

1.1.2 Drought and heat stress in plants

1.1.2.1 Drought stress

Growth rates of several plants are directly proportional to the availability of water in the soil (Kamel and Loser, 1995). Plant or cellular water deficit occur when the rate of transpiration (evaporation) exceeds water uptake resulting in the reduction of the relative water content (RWC), cell volume and cell turgor (Lawlor and Cornic, 2002). Cellular water deficit is a component of several different stresses including drought, salinity and low temperature (Bray, 1997). However, a mild water deficit emanating from drought, which is generally termed drought stress by investigators, has to be differentiated from desiccation or dehydration due to complete loss of free water, which is an extreme form of water deficit (Bray, 1997).

The plant response to drought stress depends on the species and genotype within the species, the length and severity of water loss, the age and stage of development, the

organ, cell type and also type of the sub-cellular compartment (Bray, 1997). The adaptation strategies of plants to drought stress include drought escape, drought avoidance (postponement) and drought tolerance (Levitt, 1980; Turner *et al*., 2001). Escaping drought involves completion of the life cycle before onset of the drought period. Drought avoidance involves the maintenance of the plant water status in the presence of drought stress, while drought tolerance involves maintenance of the plant function in the presence of drought. Different plant strategies to cope with drought normally involve a mixture of stress avoidance and tolerance strategies that varies with the genotype (Chaves *et al*., 2002). Under field conditions, when drought stress is imposed slowly, the early response of plants to water deficit is the closure of the stomata, which is thought to be in response to the migration of abscisic acid (ABA) synthesized in the root. This stomatal response has been linked more closely to the soil moisture content (Tardieu *et al*., 1991; Stoll *et al*., 2000).

Response to drought stress may involve metabolic and structural changes that improve plant functioning under stress (Bohnert and Sheveleva, 1998). Some of these changes include changes in root to shoot ratios, leaf anatomical changes, temporary accumulation of reserve in stem and petioles and alterations in carbon and nitrogen metabolism (Pinheiro *et al*., 2001; Chartzoulakis *et al*., 2002). Further, drought stress induces transcriptional activation of hundreds of genes, the product of some of those functions as cellular osmotic regulators under stress (Bohnert *et al*., 1995; Ingram and Bartels, 1996; Bray, 2002). Moreover, abiotic stresses, such as drought, are known to increase endogenous biosynthesis and accumulation of phytohormones like ABA and jasmonic

acid (JA). These are known to suppress expression of many photosynthetic genes including the *rbcS and rbcL* genes encoding for the small and large subunits of Rubisco. Besides phytohormone, accumulation of sugars induces changes in the expression of *rbcS* and *cab* genes encoding for the polypeptides of the light-harvesting complex (Godde, 1999).

Many environmental stresses that disrupt cellular homeostasis of cells including drought also cause the accumulation of reactive oxygen species (ROS), which are present at low concentration at normal environmental conditions. Drought stress causes closure of stomata, creating a decrease in CO_2 availability that, in turn, decreases the energy that is used for carbon fixation, causing an increase in the transit of energized electron to oxygen, creating ROS (Smirnoff, 1993). The enhanced production of ROS above the rate of its removal by detoxifying enzymes, superoxide dismutases, ascorbate peroxidases, catalases, glutathione-S-transferases and glutathione peroxidases can cause cell death through oxidative stress (Smirnoff, 1993; Noctor and Foyer, 1998; Mittler, 2002).

Drought acclimation treatments to mild or sub-lethal drought and recovery can enable the plant survive subsequent sever drought stress through limiting the accumulation of ROS and membrane lipid peroxidation (Selote *et al*., 2004). A transcriptom study in chickpea (*Cicer arientinum*) pre-treated with dehydration stress shock showed improved adaptive response during subsequent dehydration treatment due probably to maintenance for longer periods of time of certain transcripts after removal of drought stress like myoinositol-1-phosphate, (involved in synthesis of pintol) and trehalose phosphate

synthase (involved in the synthesis of trehalose), late-embryogenesis abundant groups and dehydrin (Boominathan *et al*., 2004).

1.1.2.2 Heat stress

Mid-day temperature extremes, which are above optimal for plant growth, are common in tropical environment and seasonally in temperate climate. High temperature negatively affects plant growth and survival and hence crop yield (Boyer, 1982). It has been estimated that there would be a reduction in crop yield by about 17% for each degree Centigrade increase in growing season temperature (Lobell and Asner, 2003). This is mainly due to the adverse effect of high temperature on physiological processes of the plant. High temperatures are known to affect membrane fluidity and permeability (Alfonso *et al*., 2001; Sangwan *et al*., 2002). Enzyme function is also sensitive to changes in temperature, which can lead to imbalance in metabolic pathways, or complete enzyme inactivation due to protein denaturation (Vierling, 1991; Kampinga *et al*., 1995). Photosynthesis is one of such plant processes known to be sensitive to heat stress (Crafts-Brandnerand and Salvucci, 2000). This sensitivity of photosynthesis was mainly shown to be due to decrease in the activation state of Rubisco via inhibition of Rubisco activase as shown in wheat and cotton (Law and Crafts-Brandner, 1999). Membrane and protein sensitivity/damage can lead to the production of active oxygen species that cause heat-induced oxidative stress (Dat *et al*., 1998 Larkindale and Knight, 2002). In plants, these different types of damage translate into reduced photosynthesis, impaired translocation of assimilates, and reduced carbon gain, leading to altered growth and reproduction as well as food quality (Hall, 2001; Majoul *et al*., 2003).

1.2 Plant gene expression under stress

1.2.1 Techniques to detect gene expression

Various techniques have been used for the detection of gene expression in plants (Figure 1.2). These techniques are categorized into two types. The first type involves detection of hybridization signal intensity derived from Northern blotting or a microarray, which measures relative intensity of a signal than the absolute value of the signal. The second type is based on the direct count of the individual RNA that are present in the sample, which can be achieved by "Massively Parallel Signature Sequencing" (MPSS), "Expressed Sequence Tag sequencing" (ESTs) or "Serial Analysis of Gene Expression" (SAGE). The advantages and disadvantages of these techniques are discussed in Cullis (2004). In recent years, the microarray technique has become a valuable technology in the analysis of global gene expression in response to various biotic and abiotic stresses including drought (Reymond *et al*., 2000 and 2004; Kreps *et al*., 2002; Seki *et al*., 2001, 2002a and 2002b). The DNA microarray technique allows determination of transcript abundance for many or all genes in the genome by comparing a control and with an experimental state. These studies have shown that hundreds of genes are involved in the plant drought stress response showing the quantitative nature of trait for drought resistance. Understanding the functions of these genes and their role in plant tolerance to drought stress will help improvement of drought stress tolerance of crop plants through gene transfer. Genes that are differentially expressed during drought stress have been postulated to function in adaptation to stress. However, besides regulation at transcriptional level, the translational and post-translational regulation of the gene product is noted equally important (Bray, 1997 and 2002; Kawaguchi *et al*., 2004).

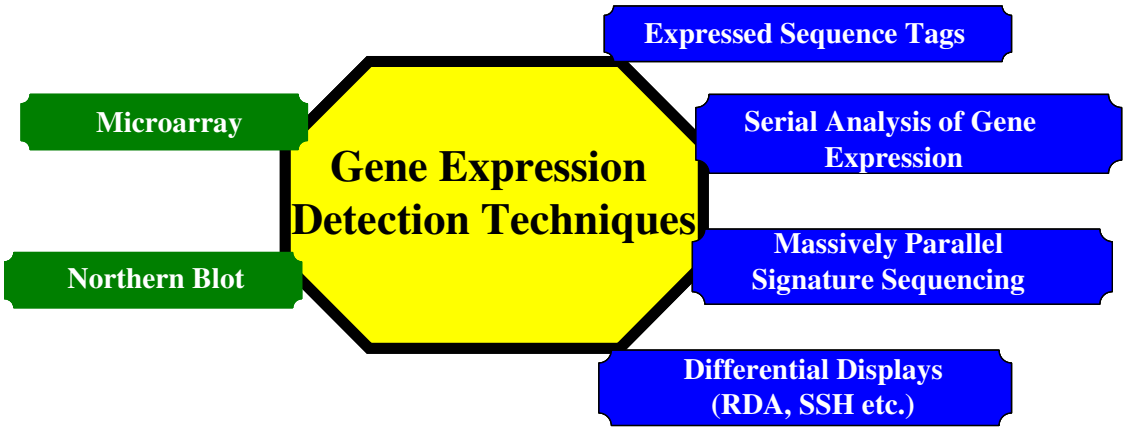


Figure 1.2 Techniques for gene expression profiling

1.2.2 Gene expression under drought stress

ABA plays important role in adapting vegetative tissues to abiotic stresses, such as drought and high salinity, and regulates the expression of many genes that might function in dehydration tolerance in both vegetative tissues and seeds (Bray *et al*., 2000; Shinozaki *et al*., 2003). But some genes that are responsive to drought stress are not regulated by ABA, which indicates that both ABA-dependent and ABA-independent regulatory systems are involved in drought-responsive gene expression. Studies on the expression of stress-regulated genes in *Arabidopsis* have shown the presence of at least four independent stress-response pathways (Figure 1.3). Two are ABA dependent (Figure 1.3, I and II) and two are ABA-independent (Figure 1.3, III and IV: Shinozaki and Yamaguchi-Shinozaki, 1997 and 2000; Yamaguchi-Shinozaki and Shinozaki, 2005). The dehydration-responsive element/C-repeat (DRE/CRT) has been identified as a *cis*-acting

element involved in one of the ABA-independent regulatory systems. Further, the t*rans*-acting factor DRE/CRT binding protein DREB1/CBF binds to this *cis*-elemnts in the promoter of drought inducible genes resulting in their activation.

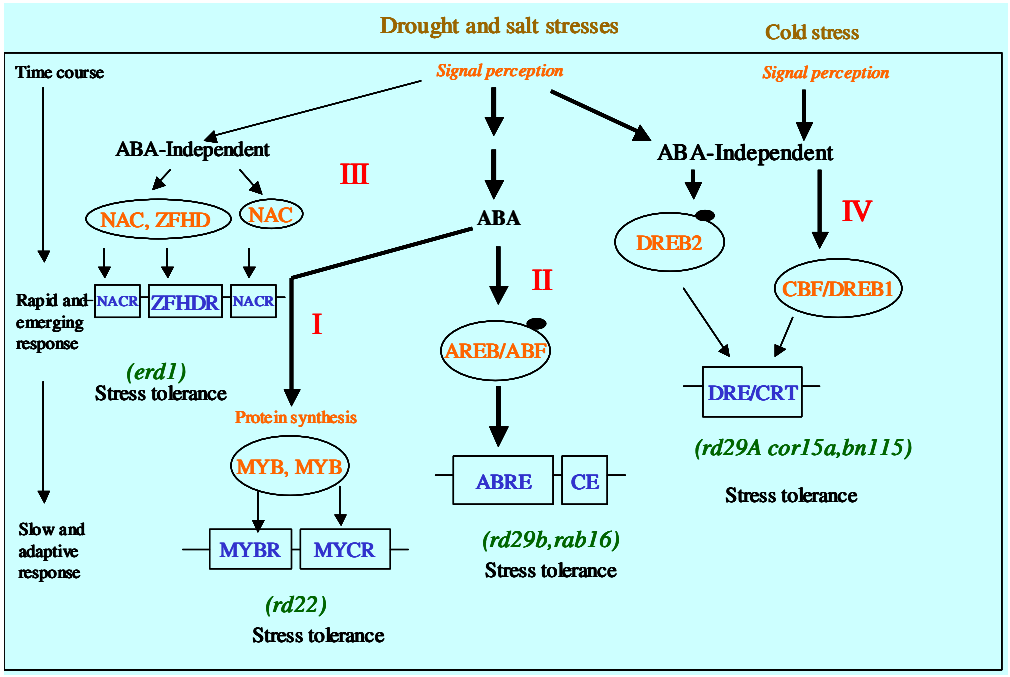


Figure 1.3 Regulatory networks of *cis*-acting elements and transcription factors involved in osmotic- and cold-stress responsive gene expression. Transcription factors controlling stress-inducible gene expression are depicted as ellipses. C*is*-acting elements involved in stress responsive transcription are depicted as coloured boxes. Small, black, filled circles reveal modification of transcription factors in response to stress signals for their activation, such as phosphorylation. Regulatory cascade of stress responsive gene expression is shown from top to bottom. Early and emergency responses of gene expression are shown in the upper part, and late and adaptive responses in the lower part. Thick black arrows indicate the major signalling pathways; these pathways regulate many downstream genes. Broken arrows indicate protein–protein interactions. Abbreviations: ABA, abscisic acid; AREB, ABRE-binding proteins; ABRE, ABA-responsive element; CBF, C-repeat-binding factor; DRE/CRT, dehydration responsive element/C-repeat; DREB, DRE-binding protein; MYBR, MYB recognition site; MYCR, MYC recognition site; NACR, NAC recognition site; ZFHDR, zinc-finger homeodomain recognition site (Redrawn from Yamaguchi-Shinozaki and Shinozaki, 2005).

Other transcriptional regulators, such as the MYC and MYB proteins, are activators in one of the ABA-dependent regulatory systems (Figure 1.3, I: Abe *et al*., 2003). ABA-responsive element functions as a *cis*-acting element in the other ABA-dependent regulatory system. ABA-responsive element binding basic leucine zipper-type proteins known as AREBs/ABFs have been identified as transcriptional activators in this ABA-dependent regulatory system (Figure 1.3, II: Choiet *et al*., 2000; Uno *et al*., 2000).

Based on their temporal responses and function, drought-inducible genes are classified in to two major categories, those which are directly involved in stress tolerance (also called functional genes) and those which are involved in regulation of gene expression and signal transduction in stress response (also called regulatory genes) (Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Yamaguchi-Shinozaki *et al*., 2002; Seki *et al*., 2002a and 2002b). Genes that respond early after recognition of stress are more likely involved in the signal transduction pathway and have a regulatory role over down- stream responsive genes. The first group called functional proteins (Figure 1.2) that are directly involved in stress tolerance include: (i) proteins that directly protect macromolecules like enzymes lipids and mRNA from dehydration, these are late embryogenesis abundant proteins (LEA), chaperones and mRNA binding proteins; (ii) compatible solutes like proline, glycine betain and sugars which functions as osmolytes and protect cells from dehydration; (iii) water channel proteins, sugar transporters and proline transporters which function in transport of water, sugars and proline through plasma membranes and tonoplast to adjust the osmotic pressure under stress conditions; (iv) detoxifying enzymes, such as glutathione S-transferase, superoxide dismutase and a

soluble epoxide hydrolase which are involved in protection of cells from active oxygen species and (v) proteinases and proteinase inhibitors, which determine protein degradation. The second group of gene contains protein factors involved in regulation of signal transduction and gene expression (Figure 1.4) that probably function in stress response and includes protein kinases, transcription factors and enzymes in phospholipids metabolism (Yamaguchi-Shinozaki *et al*., 2002).

Genes for a variety of transcription factors that contain typical DNA binding motifs, such as basic-domain leucine zipper (bZIP or AREB1), MYB, MYC, ERF/AP2, and Zinc fingers, and various protein kinases, such as MAP kinases, calcium dependent protein kinases (CDPK), SNF1 related protein kinase and ribosomal S6 kinases have been found to be induced by drought stress (Seki *et al*., 2002a and 2002b for reviews: Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki *et al*., 2002; Bray, 1997 and 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). These transcription factors function in regulation of various functional genes in response to abiotic stress. Drought stress-induced protein kinases and phosphatases have been suggested to be involved in modification of functional proteins and regulatory proteins involved in stress signalling pathways.

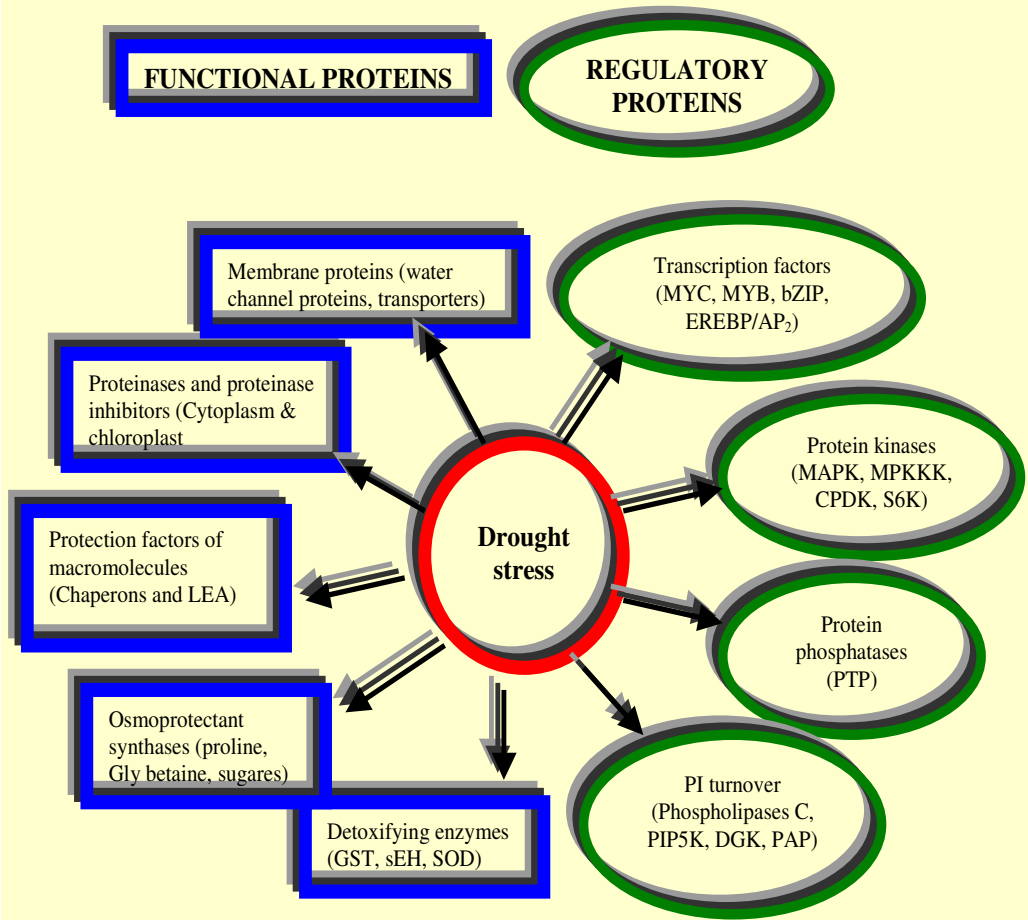


Figure 1.4 Drought stress inducible genes and their possible functions in stress tolerance and response. Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (functional proteins), and the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (regulatory proteins) (Adapted from Yamaguchi-Shinozaki *et al*., 2002).

1.2.3 Gene expression under heat stress

Plants exposed to excess heat exhibit a characteristic set of cellular and metabolic responses, including a decline or cessation of housekeeping proteins and an accelerated accumulation of wide array of stress inducible proteins. This includes small protein groups called heat shock proteins (HSPs) (Guy, 1999). These proteins act as molecular chaperones to protect cellular proteins against irreversible heat-induced denaturation and to facilitate refolding of heat-damaged proteins (Boston *et al*., 1996; Iba, 2002). Thermo-inducibility of HSP genes is regulated by heat-dependent activation of heat shock factors (HSF) that recognize and bind to a heat shock element (HSE) in the promoter of HSP genes (Iba, 2002; Wang *et al*., 2003). A recent study using the 21 *Arabidopsis* HSF, showed that not all HSFs were induced under heat stress, since there are also light, oxidative stress, drought and heat stress specific HSFs (Pnueli *et al*., 2003; Rizhsky *et al*., 2004). Transcripts of genes related to mitochondrial proteins, such as NADH dehydrogenase and cytochrome c oxidase, that are related to increased heat stress induced respiration and many ROS-scavenging enzymes were found to be elevated by heat stress (Rainwater *et al*., 1996, Rizhsky *et al*. 2002 and 2004; Vacca *et al*., 2004). Varietal sensitivity or tolerance to heat stress has been also shown to depend on the ability to maintain activities of antioxidant enzymes (Rainwater *et al*., 1996; Dash and Mohanty, 2002). The heat tolerant genotype of fescue maintained higher transcripts of genes involved in cell maintenance, chloroplast associated and photosynthesis-, protein synthesis-, signalling-, and transcription factor-related genes. In contrast, genes related to metabolism and stress had higher expression in the heat-sensitive genotype (Zhang *et al*., 2005).

1.3 Plant engineering for drought and heat stress tolerance

1.3.1 Plant engineering for drought stress

The genetic engineering of plants for enhanced drought stress tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. Available strategies employ the transfer of one or several genes that are either involved upstream in the drought stress response cascade. This includes signaling and regulatory genes or their down-stream target genes. Such genes include signal sensors/transducers, transcription factors/co-activators, compatible solutes, antioxidants and detoxifying enzymes, ion transport, heat shock proteins and molecular chaperones and late embryogenesis abundant proteins (Figure 1.5). Table 1.1 gives a list of mechanisms, genes, transformed plants and the enhanced tolerance (for detailed reviews see Bajaj *et al*., 1999; Zhang *et al*., 2000; Zhu, 2002; Iba, 2002; Chen and Murata, 2002; Wang *et al*., 2003). Abiotic stresses, such as drought, cold, salt and heat, are usually interrelated and induce a similar set of plant responses (genes) by activating similar signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Reymond *et al*., 2000 and 2004; Kreps *et al*., 2002; Seki *et al*., 2001, 2002a and 2002b). Overexpression of a particular gene(s) might thus result in cross-tolerance to multiple stresses. For example, transgenic *Nicotiana tabacum* plants overexpressing a key enzyme in proline biosynthetic pathway (P5CS) were tolerant to drought and salt (Kishor *et al*., 1995) as well as cold (Konstantinova *et al*., 2002) and oxidative stresses (Hong *et al*., 2000).

The use of molecular switches (regulatory genes) that regulate a number of down-stream drought responsive genes seems a promising approach in the development of drought

resistant/tolerant transgenic plants when compared to engineering of individual functional genes. Overexpressed DREB1A in Arabidopsis driven by either a constitutive (CaMV 35S) or dehydration-inducible (rd29A) promoter has resulted in increased tolerance to freezing, salinity and drought (Kasuga *et al*., 1999) and also increased expression of down-stream stress-inducible target genes. In similar studies, overexpression of Arabidopsis DREB1A in *Nicotiana tabacum* has increased drought and freezing tolerance and enhanced expression of LEA-type genes (Kasuga *et al*., 2004). Overexpression of Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to drought/salinity and drought by activating expression of 12 (CBF3/DREB1) and 7 (ABF3) target genes, respectively, under non stress condition. In addition, 13 and 27 genes were activated under stress (Oh *et al*., 2005) without any growth retardation or visible phenotypic effects. Transgenic rice and Arabidopsis plants overexpressing *Oryza sativa* OsDREB1 or *Arabidopsis thaliana* DREB1 genes showed improved tolerance to drought, high-salt and low-temperature stresses and also accumulated elevated levels of osmoprotectants such as free proline and various soluble sugars (Ito *et al*., 2006). Target genes of the DREB1A/CBF3 included transcription factors, phospholipase C, RNA-binding protein, sugar transport protein, desaturase, carbohydrate metabolism-related proteins, late embryo abundant (LEA) proteins, KIN (cold-inducible) proteins, osmoprotectant biosynthesis proteins, proteinase inhibitors (Seki *et al*., 2001, Fowler and Thomashow 2002, Maruyama *et al*., 2004, Vogel *et al*., 2005; Oh *et al*., 2005; Ito *et al*., 2006). Besides transcription factors, protein phosphorylation and dephosphorylation plays a major role in signaling events induced by drought stress (Bray, 2002; Yamaguchi-Shinozaki *et al*., 2002). Constitutive overexpression of *Arabidopsis* SNF1-related protein

kinase 2 (SnRK2), SRK2C, in *Arabidopsis* revealed higher overall drought tolerance than control plants. This was coincided with upregulation of many stress responsive genes like RD29A, COR15A, a transcription factor DREB1A/CBF and a portion of its target genes (Umezawa *et al*., 2004).

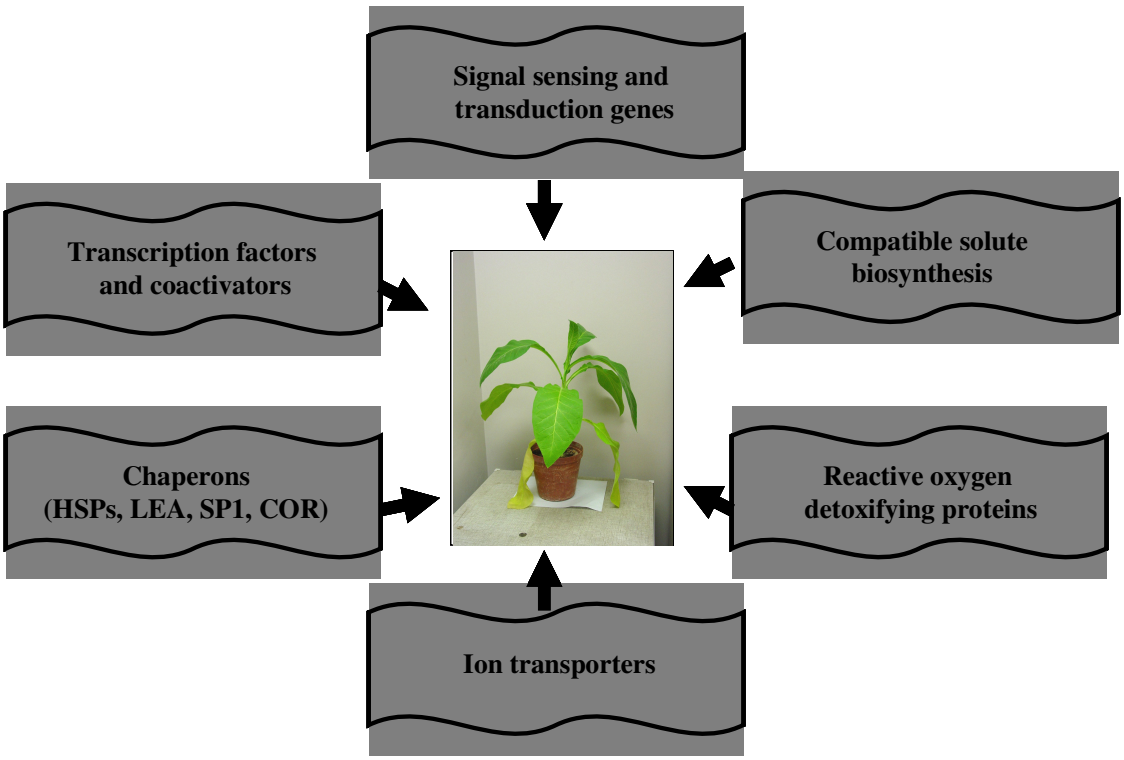


Figure 1.5 Genes that have been used to enhance abiotic stress tolerance in plants.

It is conceivable that unlike most monogenic traits of engineered resistance to pests and herbicides, the genetically complex responses to drought stress, which is multigenic in nature, has made it difficult in delivering transgenic crops that are tolerant to drought to farmers (Bajaj *et al*., 1999; Iba, 2002; Wang *et al*., 2003). In some of these transgenic plants, unforeseen side effects have been found such as toxicity or stunted growth by plants expressing compatible solutes like sorbitol (Sheveleva *et al*., 1998).

Overexpression of the transcription factor DREB1/CBF driven by the strong constitutive promoter has resulted in growth retardation of transgenic plants under unstressed normal growth in *Arabidopsis*, rice and tobacco (Liu *et al*., 1998, Kasuga *et al*., 1999; 2004; Ito *et al*., 2006). This was alleviated by using a stress specific promoter such as rd29A (Kasuga *et al*., 1999 and 2004) (for review see Chen and Murata, 2002; Wang *et al*., 2003). The significant achievements in discovery of genes that are responsive to drought stress and the functional analysis and understanding of the upstream regulatory elements and cross-talks among them will help to engineer agriculturally important plants that can withstand drought stress (Cushman and Bohnert, 2000).

Table 1.1 Mechanisms, genes, genetically modified plants and targeted abiotic stress (adapted from Wang *et al*., 2003).

Mechanism	**Genes**	**Transformed plants**	**Enhanced tolerance**	**Reference**
Signal sensing/ transduction	NPK1	Z. mays	Drought	Shou et al., 2004
	SRK2C	A. thaliana	Drought	Umezawa et al., 2004
	OsCDPK7	O. sativa	Drought/salt and freezing	Saijo et al., 2000
	CBL1	A. thaliana	Drought and salt	Cheong et al., 2003
Transcription control	CBF1	*A. thaliana*	Freezing	Jaglo-Ottosen et al. 1998
	DREB1	*A. thaliana*	Drought, salt and freezing	Kasuga et al., 1999
		T. aestivum	Drought	Pellegrineschi et al., 2004
	CBF3	*A. thaliana*	Freezing	Gilmour et al., 2000
		O. sativa	Drought and salt	Oh et al., 2005
	ABF3	*O. sativa*	Drought	Oh et al., 2005
	ABF3/ABF4	*A. thaliana*	Drought	Kang et al., 2002
	CBFs	*B. napus*	Freezing	Jaglo et al., 2001
	CBF1	*L. esculentum*	Chilling and oxidative stress	Hsich et al., 2002
	CBF4	*A. thaliana*	Freezing and drought	Haake et al., 2002
	AtMYC2 & AtMYB2	*A. thaliana*	Drought	Abe et al., 2003
	AREB1	*A. thaliana*	Drought	Fujita et al., 2005
	HSF1 or HSF3	*A. thaliana*	Heat	Lee et al., 1995 Prandl et al., 1998
	HsfA1	*L. esculentum*	Heat	Mishra et al., 2002
	14 -3 -3	*G. Hirsutum*	Drought	Yan et al., 2004
	MBF1c	*A. thaliana*	Drought, heat and osmotic stress	Suzuki et al., 2005
	spl7	*O. sativa*	Heat	Yamanouchi et al., 2002
Compatible solutes				
Proline	P5CS	*N. tabacum*	Drought and salt	Kishor et al. 1995; Konstantinova et al. 2002; Hong et al. 2000
	ProDH	*A. thaliana*	Freezing and salt	Nanjo et al., 1999
Myo-inositol	IMT1	*N. tabacum*	Salt and drought	Sheveleva et al., 1997
Sorbitol	*stpd1*	*N. tabacum*	Salt and drought	Sheveleva et al., 1997

Glycinebetaine	*codA*	*A. thaliana*	High temperature	Alia et al., 1998
		A. thaliana	Salt and cold	Alia et al., 1997
	betA	*Z. mays*	Drought	Quan et al., 2004
Antioxidants and detoxification	CuZn-SOD	*N. tabacum*	Oxidative stress	Gupta et al. 1993a, 1993b; Pitcher and Zilinskas 1996
	Mn-SOD or Fe-SOD	*M. sativa,* *N. tabacum*	Oxidative stress	McKersie et al. 1996, 1999, 2000; Van Camp et al. 1996
	GST and GPX	*N. tabacum*	Oxidative stress	Roxas et al., 1997
	chyB	*A. thaliana*	Oxidative stress	Davison et al., 2002
	Aldose-aldehyde reductase	*N. tabacum*	Oxidative stress	Oberschall et al. 2000
Ion transport	*AtNHX1*	*A. thaliana*	Salt	Apse et al. 1999
		B. napus	Salt	Zhang et al. 2001
		L. esculentum	Salt	Zhang and Blumwald 2001
		G. hirsutum	Salt	He et al., 2005
	SOS1	*A. thaliana*	Salt	Shi et al., 2003
	HAL1	*C. melo*	Salt	Bordas et al. 1997
		L. esculentum	Salt	Rus et al. 2001
	AVP1	*A. thaliana*	Salt and drought	Gaxiola et al. 2001
		L. esculentum	Drought	Park et al., 2005
Hsps and molecular chaperones	Hsp17.7	*D. carota*	Heat	Malik et al., 1999
	Hsp21	*A. thaliana*	Oxidative stress	Härndahl et al., 1999
	AtHSP17.6A	*A. thaliana*	Salt and drought	Sun et al., 2001
	DnaK1	*N. tabacum*	Salt	Sugino et al., 1999
	SP1	*P. tremula*	Salt	Wang et al., 2003
	Hsp101	*O. sativa*	Heat stress	Katiyar-Agarwal et al., 2003
LEA-type proteins	COR15a	*A. thaliana*	Freezing	Artus et al. 1996; Steponkus et al. 1998; Jaglo-Ottosen et al. 1998
	HVA1	*O. sativa*	Salt and drought	Xu et al., 1996
		T. aestivum	Drought	Sivamani et al., 2000
	WCS19	*A. thaliana*	Freezing	Ndong et al., 2002

1.3.2 Plant engineering for heat stress

The complex and multigenic nature of heat stress tolerance has been shown recently. *Arabidopsis* mutants deficient in ethylene, ABA, ROS, and SA-signaling pathways, including knockouts for the respiratory burst oxidase enzyme RbohD, showed strong defects in acquired heat tolerance. This suggests the essential roles of these pathways in acquired heat tolerance (Larkindale *et al*., 2005, Suzuki *et al*., 2005). Engineering for heat stress tolerance not only involves HSP and HSF, but also different genes involved in different mechanism of stress tolerance pathways. HSP100 family proteins are essential for the acquisition of thermotolerance in plants. Loss-of-function mutants of HSP101 in Arabidopsis *hot1* (Hong and Vierling, 2000 and 2001) and maize (Nieto-Sotelo *et al*., 1999) were unable to acquire thermotolerance at several different growth stages. While transgenic *Arabidopsis* over-expressing HSF3 showed higher activity of ascorbate

peroxidase (APX) during post heat-stress recovery and had a much stronger induction of APX2 than wild type plants (Panchuk *et al*., 2002). Overexpression of HSF1 and HSF3 (class A) leads to the expression of several HSP genes conferring thermo-tolerance in transgenic *Arabidopsis* plants (Lee *et al*., 1995; Prändl *et al*., 1998). In tomato plants, overexpression of HSFA1 resulted in heat stress tolerance. In contrast HSFA1 antisense plants and fruits were extremely sensitive to elevated temperatures (Mishra *et al*., 2002). Similarly, transgenic rice engineered to overexpress *Arabidopsis* HSP101 showed better growth performance following heat stress treatment than a corresponding control (Katiyar-Agarwal *et al*., 2003). *Arabidopsis* plants overexpressing carrot HSP 17.7 (Malik *et al*., 1999) choline oxidase (*codA*) for enhanced accumulation of glycine betain (Alia *et al*., 1998) were tolerant to heat stress. Davison *et al*. (2002) showed that overexpression of the *chyB* gene, which encodes beta-carotene hydroxylase, an enzyme in the zeaxanthin biosynthetic pathway conferred tolerance to high light and high temperature stresses in *Arabidopsis thaliana*. It was assumed that such a protection was due to the function of zeaxanthin in preventing oxidative damage of membranes.

1.4 Proteinase/proteinase inhibitor system and stress

1.4.1 Plant proteinases

The *Arabidopsis thaliana* genome is estimated to contain over 550 proteinase sequences representing all the five catalytic types: serine, cysteine, aspartic acid, metallo and threonine (MEROPS, peptidase database, http://merops.sanger.ac.uk/) (Beers *et al*., 2004). Proteinases are required for a broad range of genetically programmed and inducible processes in addition to their classical roles in starvation, stress response and nutrient mobilization. Recent findings revealed that certain serine, cysteine and aspartic proteinases are required in plant growth and development events such as stomatal distribution, embryo development, and disease resistance (Beers *et al*., 2004). The term proteinase (peptide hydrolase or peptidases) comprises two groups of enzymes, the endo-peptidases, which act on the interior of the peptide chain, and exopeptidases, which cleave peptide bonds on the termini of the peptide chains. The latter is differentiated according to their substrate specificities as amino-peptidases, which are able to cleave peptides at the N-terminus, and carboxy-peptidases, which degrade peptides at the C-terminus (Barrett, 1994). Endo-peptidases (proteinases) are classified according to the amino acid residue in their reactive site as serine, cysteine, aspartic and metallo-proteinases and probably threonine proteinases.

1.4.1.1 Cysteine proteinases

Plant cysteine proteinases are involved in diverse range of plant processes (Figure 1.6), including processing and proper folding of storage proteins during seed development (Gruis *et al*., 2002; Shimada *et al*., 2003), remobilisation of stored proteins to supply

amino acids for synthesis of new proteins during seed germination and senescence and in developmental and stress-induced programmed cell death (PCD). Cysteine proteinases are involved in remobilisation of stored proteins during seed germination to provide amino acids to germinating seedlings. They are the most abundant group of proteinases responsible for degradation and mobilization of storage proteins (Grudkowska and Zagdańska, 2004). In germination of barley seeds 42 proteinases are involved and among them 27 are cysteine proteinases (Zhang and Jones, 1995). In other cereals cysteine proteinases account for over 90% of the total degradation activity of prolamins, the major storage proteins of cereals, in germinating maize (de Barros and Larkins, 1994) and wheat (Bottari *et al*., 1996). In the cotyledon of certain germinating dicot seeds, papain- (SH-EP, CPR1, CPR2, CPR4, proteinas A) and legumain-like (VsPB2 and proteinase B) proteinases were shown to be involved in protein remobilisation (Okamoto and Minamikawa, 1998; Fischer *et al*., 2000; Schlereth *et al*., 2001; Tiedemann *et al*., 2001). Sprouting mature potato tubers rely exclusively on cysteine proteinases for protein mobilization during germination (Michaud *et al*., 1994).

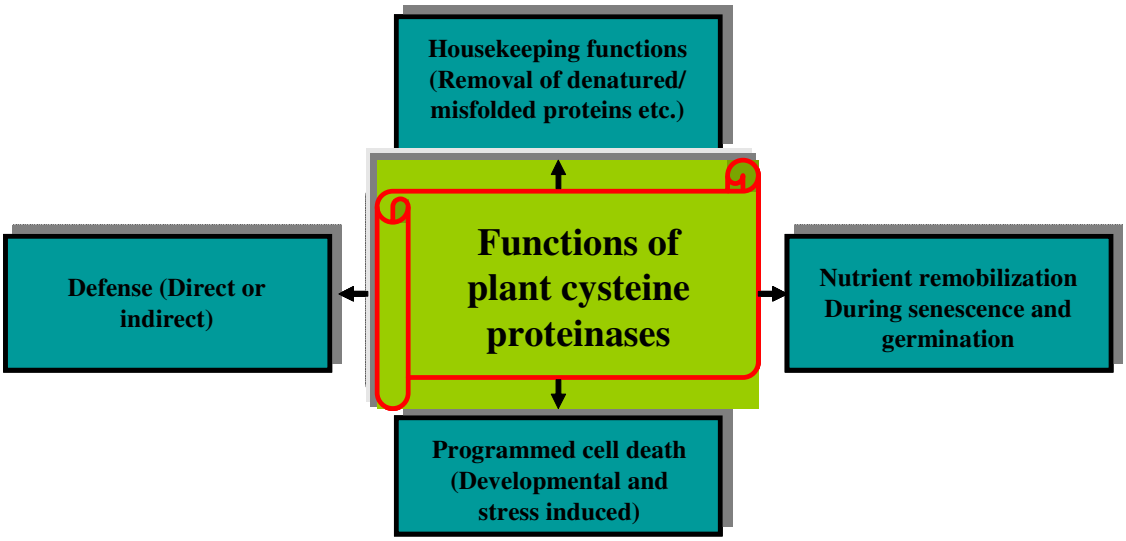


Figure 1.6 Functions of plant cysteine proteinases.

Degradation of protein during leaf senescence is an important phenomenon by which leaf N is recycled. Nitrogen is primarily released from protein breakdown and by nucleic acids metabolism (Hortensteiner and Feller, 2002) and N was estimated to be the most recycled nutrient (90%) during senescence (Himelblau and Amasino, 2001). Proteolysis requires the involvement of proteinases of which cysteine proteinases are the major executors of protein degradation in senescing leaves (Guo *et al*., 2004). In a recent study of *Arabidopsis* leaf senescence, a total of 116 genes were predicted to be involved in proteolysis during senescence. This represents 7% of total leaf senescence ESTs in *Arabidopsis* (Guo *et al*., 2004). Of these genes involved in proteolysis, 75 genes, which account for about 38% protein degradation according to digital northern estimates from ESTs, are associated with the ubiquitin-proteolysis pathway. Thirty five genes are proteinases of which cysteine proteinases account for 57% of total proteolysis and the remaining 5-6% accounted for by proteinases including serine, aspartic and other peptidases (Guo *et al*., 2004). In their study, eight cysteine proteinase genes were identified to be involved in senescence of which four proteinases SAG12 (At5g45480), AALP (At5g60360), Cathepsin B-like proteinase (At4g01610) and cysteine proteinase like protein (At4g16190) had the most abundant ESTs counts of 136, 42, 22 and 15, respectively. This represents over 50% of the total ESTs known to be involved in proteolysis. This shows that cysteine proteinases are indeed very essential in nutrient remobilisation in senescing leaves including PCD (Gan and Amasino *et al*., 1997). Despite such an abundant amount of a cysteine proteinase SAG12 in senescing *Arabidopsis* leaves, mutation in SAG12 gene did not result in an altered senescence phenotype. This indicates that SAG12 is not required for visual progression of

senescence (Otegui *et al*., 2005). In *Brassica olerace*, suppression of (through antisense technology) senescence induced aleurain (an *Arabidopsis* orthologue of AALP/SAG2) in floret and leaves delayed post-harvest floret senescence (Eason *et al*., 2005).

The involvement of cysteine proteinases in leaf and flower senescence has been investigated by a number of researchers using different plant species including *Arabidopsis*. Senescence-enhanced increase of cysteine has been found in leaves of tomato (Drake *et al*., 1996) *Brassica napus* (Buchanan-Wollastan and Ainsworth, 1997), maize (Smart *et al*., 1995), tobacco (Ueda *et al*., 2000) and flowers of daylily (Guerrero *et al*., 1998), pea (Cercos *et al*., 1999), Alstroemerria (Wagstaff *et al*., 2002), sandersonia (Eason *et al*., 2002) and ripening fruits of citrus (Alonso and Granell, 1995). Other large-scale transcriptom studies of senescing autumn leaves of Aspen (*Populus tremula*) (Bhalerao *et al*., 2003) senescing leaves of *Arabidopsis thaliana* (Gepstein *et al*., 2003) and senescing cultured cells of *Arabidopsis* representing PCD (Swidzinski *et al*., 2002) also showed abundance and expression of cysteine proteinases.

PCD in multi-cellular organisms occurs as a part of normal development and is one of the plant defense mechanisms against biotic and abiotic stresses. In plants, PCD occurs during developmental changes and differentiation of plant organs. This is associated with induction of cysteine proteinases, such as the process of xylogenesis in *Zinnia*, which leads to formation of vascular tissues (Minami and Fukuda, 1995) and differentiation of tracheary elements in *Arabidopsis* (Funk *et al*., 2002). In germinating castor bean (*Ricinus cummunis*), seed PCD in the endosperm is associated with the accumulation of a

KDEL-tailed 45 kDa papain-like pro-peptidase (CysEP) in endoplasmic reticulum-derived structures called ricinosomes. The release of the mature 35 kDa form of CysEP from ricinosomes occurs during cell collapse after mobilization of stored proteins to the developing cotyledons has occurred (Schmid *et al*., 1999). It was further proposed that a similar mechanism could also operate in other plant species and organs because homologous KDEL-tailed proteinases have been identified in several senescing tissues. This includes withering daylily petals and drying seed coats (Gietl and Schmid, 2001) as well as white spruce (*Picea glauca*) megagametophyte seeds (He and Kermode, 2003). Moreover, three genes for KDEL-tailed cysteine proteinases (CEP1, CEP2 and CEP3) were also identified from *Arabidopsis* and were localized in senescing ovules, vascular vessels and maturing siliques, which might represent organs undergoing PCD (Gietl and Schmid, 2001). In *Brassica napus*, a papain like cysteine proteinase BnCysP1 is associated with PCD of the inner integument of the seed coat during early stages of seed development (Wan *et al*., 2002). A brinjal (*Solanum melongena*) cysteine proteinase SmCP has been identified to be involved in PCD during xylogensis, anther senescence and ovule development (Xu and Chye, 1999). A detailed account of forms of developmental PCD occurring in plants have been presented in an excellent review of van Doorn and Woltering (2005).

Recent studies have revealed that cysteine proteinases known as legumains, which are also called vacuolar processing enzyme (VPE) or asparaginyl endopeptidases, have caspase-1 activity. VEP shares several enzymatic properties with caspase, which is a cysteine proteinase that is involved in animal PCD, although VPE is not related to the

caspase family or the meta-caspase family (Hara-Nishimura *et al*., 2005). Plant VPE homologs can be separated into two groups in *Arabidopsis*: (1) vegetative VPEs (αVPE and γVPE) and (2) seed type VPEs (βVPE) (Kinoshita *et al*., 1999). By analysing VPE-deficient *Arabidopsis* mutants, it was shown that the seed-type βVPE is essential for proper processing of storage proteins because a triple VPE-deficient mutant (lacking α, β and γVPE) accumulated unprocessed pro-proteins in seeds (Shimada *et al*., 2003). In contrast, vegetative-type αVPE and γVPE are up-regulated in association with various types of PCD. This includes leaf senescence in cortex cells adjacent to the emerging lateral roots and in vascular tissues and under stress conditions (Kinoshita *et al*., 1999; Hara-Nishimura and Maeshima, 2000).

In a similar study, the involvement of tobacco VPEs in tobacco mosaic virus-induced hypersensitive (HR) cell death was found (Hatsugai *et al*., 2004). The authors found that VPE-deficient *Nicotiana benthamiana* leaves have no visible lesions upon TMV-infection. The VPE appeared rapidly at the beginning of the HR and declined before appearance of lesions. VPE as a vacuolar enzyme plays an essential role in the regulation of lytic system because VPE deficiency suppresses the disintegration of the vacuolar membranes in the TMV-infected leaves. This shows that the VPE is involved in vacuolar collapse, which triggers hypersensitive cell death. It was suggested that VPE could mediate the initial activation of some of the vacuolar enzymes and/or the disruption of the vacuole membrane (Hara-Nishimura *et al*., 2005). Such a mechanism is distinct from animal PCD, where caspases are localized in cytosol and triggers a death cascade. Further, VPEs trigger vacuolar collapse, which in turn results in cell suicide. Plant might

have evolved a regulated cellular suicide strategy, which unlike animal apoptosis, is mediated by VPEs and the vacuoles (Hatsugai *et al*., 2004; Yamada *et al*., 2005; Hara-Nishimura *et al*., 2005). A fourth VPE from *Arabidopsis,* δVPE, has been further shown to be involved in developmental PCD during embryogenesis (Nakaune *et al*., 2005) to form the seed coat.

Papain-like cysteine proteinases and their inhibitors have been found to be involved as modulators of PCD induced by biotic and abiotic factors. In a first report, Solomon *et al*. (1999) showed that PCD in soybean suspension cultures activated by oxidative stress induced expression of a set of cysteine proteinases. The proteinases were inhibited by ectopic expression of a cystatin, an endogenous cysteine proteinase inhibitor (PI) gene, without extensively affecting constitutive proteinase. This blocked PCD, which was triggered by an avirulent pathogen *Pseudomonas syringae* pv *glycinea* or oxidative stress. In a similar study, Belenghi *et al*. (2003) demonstrated that *Arabidopsis* cystatin AtCYS1, which is constitutively expressed in roots and siliques of *A. thaliana*, was wound, pathogen or nitric oxide inducible in leaves. Overexpression of AtCYS1 blocked cell death triggered by either avirulent pathogens or by oxidative and nitrosative stress in both *A. thaliana* cell suspension and in transgenic tobacco leaves overexpressing AtCYS1. These studies demonstrated the involvement of papain-like cysteine proteinases as executors of PCD. Although the presence of caspase-like activities in tissues undergoing PCD was not tested and the actual cysteine proteinase involved in the process of PCD was not cloned, the abolition of PCD by a cystatin indicates that plant PCD could take a different course than animal PCD, where caspases are involved. Also caspases and

VPEs are both not inhibited by well-known inhibitors of papain-type cysteine proteinases such as E-64 (*trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane), leupeptin or antipain. VPEs are inhibited by type II cystatins (egg-white and human cystatin C) and type III cystatins (kininogen; Abe *et al*., 1993; Rotari *et al*., 2001; Outchkourov *et al*., 2003), but not by potato cystatin (PhyCys) and stefin A (Outchkourov *et al*., 2003).

1.4.1.2 Cysteine proteinase expression under drought and heat stress

Abiotic stresses induce a number of cysteine proteinases, which are involved in various functions like degradation of proteins denatured by physiological stress and proteolytically activation of specific proteins. This may then function in intracellular adaptation to the stress or amino acid metabolism (Stroeher *et al*., 1997; Jones and Mullet, 1995). Their induction during drought stress might be a result of oxidative stress (Bray, 2002). Table 1.2 lists abiotic and biotic stresses inducible proteinases and (PIs). Salt and dehydration stresses have been found to induce a pea cysteine proteinases *Cyp15a* (Jones and Mullet, 1995) and two distinct *Arabidopsis* proteinases *rd19A* and *rd21A* (Koizumi *et al*., 1993). A *Brassica napus* proteinases *bcp-15* (Stroeher *et al*., 1997) and *Arabidopsis* A1494 (Williams *et al*., 1994) were induced by drought and low temperature and moderately by heat shock. The expression of a barley cathepsin B-like cysteine proteinase, which was ubiquitously present in different organs increased in leaves by cold shock and was suppressed by dark treatment (Martinez *et al*., 2003). In *Zea mays*, a cysteine proteinase (SEE1) was induced in naturally senescing leaves during seedling germination and chilling stress treatment in lines tolerant to chilling stress but

decreased in sensitive lines. The mRNA abundance also decreased during dark-induced senescence and in nutrient and water-stressed treatments (Griffiths *et al*., 1997).

Harvest-induced wilting and senescence of *Brassica oleracea* florets results in induction of four dehydration-responsive cysteine proteinases BoCP1, BoCP2, BoCP3 and BoCP4 (Coupe *et al*., 2003). Some of the dehydration inducible proteinases, like RD21A, AALP (SAG2), and At4g16190 are also senescence-associated proteinases and their expression during drought stress might indicate their indirect response to stress-induced senescence, since drought stress is known to enhance leaf senescence (Pic *et al*., 2002). A two dimensional gel electrophoresis analysis of water-stressed lupin (*Lupinus albus*) stems have shown that serine and cysteine proteinases and their inhibitors (PIs) were the major identified proteins. Re-watering of stressed plants did not cause *de novo* expression of proteins but increased the expression level of PIs (Pinheiro *et al*., 2005). The authors suggested that severe water stress led to the expression of proteinases that are engaged in selective protein processing of some unidentified regulatory mechanisms. The PIs might modulate proteinase activities particularly relevant during re-watering.

Table 1.2 outlines stresses and growth regulator (ABA and JA or methyl jasmonate, MeJA)-induced proteinases and inhibitors. Most of the data are obtained from large-scale transcriptome studies using the microarray technique. In *Arabidopsis*, two cysteine proteinases At4g39090 (rd19A) and At4g16190 were induced by drought (Seki *et al*, 2001) or ABA (Hoth *et al*., 2002). In rice, a papain like cysteine proteinase AK073373 was induced by cold, drought and high salinity stress and also ABA application and the

proteinase contained a *cis*-acting DRE-element in its promoter sequence (Rabbani *et al*., 2003). Further, a cathepsin B-like cysteine proteinase At4g01620 was induced by drought and ABA and a cysteine proteinase At3g19390 (Seki *et al*., 2002b) was induced by ABA application.

Induction of cysteine proteinases during drought stress has been associated in wheat with cultivar differences in resistance to drought. Cysteine proteinases were induced and their activity increased significantly during drought stress in drought-acclimated and non-acclimated wheat (Zagdańska and Wiśniewski, 1996). Cultivars having different drought resistance revealed that the level of cysteine proteinase induction was negatively related to the drought resistance and positively correlated with extravacuolar ATP-dependent proteolysis (Wiśniewski and Zagdańska, 2001). This may indicate that, unlike cysteine proteinase induction, the inducibility of PIs is associated with drought tolerance (Diop *et al*., 2004; Riccardi *et al*., 2004).

Cysteine proteinases are also induced during nutrient deficiency. A short period of sulfur deprivation in tobacco (*Nicotiana tabacum*) has resulted in accumulation of a proteinase transcript homologous to senescence-enhanced NTCP-23. In addition, a phytocystatin (PhyCys) gene homologous to tomato STC (AF198389) was down-regulated in sulfur-deficient tobacco (Wawrzyńska *et al*., 2005). In a similar study, a drought-inducible *A. thaliana* gene (At4g16190) encoding a cysteine proteinase was up-regulated after both short-term (Hirai *et al*., 2003) and long-term (Nikiforova *et al*., 2003) sulfur-limitation. Nitrogen limitation has resulted in increased activation of cysteine proteinases without

affecting total proteolytic activities in leaves of white clover. Kingston-Smith *et al*. (2005) showed that proteinases involved in remobilisation during nutrient limitations were distinct from those involved during the natural senescence. Further, a unique drought-inducible cysteine proteinase of *Arabidopsis* (At4g16190) has been found when compared to other proteinases. It is induced under various abiotic stress conditions, including drought (Seki *et al*., 2001), phosphorus starvation (Hammond *et al*., 2003), sulfur deficiency (Nikiforova *et al*., 2003), sucrose starvation (Contento *et al*., 2004) and ABA treatment (Hoth *et al*., 2002). Such induction by various stresses might indicate the virtual importance of the enzyme in adaptation of the cellular metabolism to various stresses.

Besides induction of PIs, wounding also induces accumulation of all four mechanistic classes of proteinases (Ryan, 200). A cathepsin B-like cysteine proteinase from *Nicotiana rustica* and *Nicotiana tabacum* (Lidgett *et al*., 1995), the papain-like cysteine proteinases NTCYP7 and NTCYP8 (Linthorst *et al*., 1993), the *Arabidopsis* αVPE and γVPE (Kinoshita *et al*., 1999) and the subtilisin-like protease AF055848 (Cheong *et al*., 2002) were induced by wounding in leaves. In contrast, a tobacco cysteine proteinase NTCP-23 (Ueda *et al*., 2000), two *Arabidopsis* serine carboxypeptidases (AC004401 and AC006929) and a cysteine proteinase (Z97340) were down-regulated in response to mechanical wounding (Cheong *et al*., 2002). The exact role of most of proteinases reported as responsive to wounding is still unclear; the existing literature presents only speculative ideas. But in a few instances, such as maize *mir1,* the direct involvement of the proteinase against lepidopteran attack has been established (Pechan *et al*., 1999, 2000

and 2002). A papain-like 33-kDa cysteine proteinase, *mir1* of maize AF019145 (Pechan *et al*., 1999), was up-regulated in resistant genotypes of maize lines due to lepidopteran predation. The abundance of the proteinase increased dramatically upon larval feeding and by wounding. In addition, it was also up-regulated developmentally in senescing leaves and in non-friable callus tissues (Pechan *et al*., 2000). The proteinase significantly reduced larval growth in resistant genotypes. Ectopic expression of this proteinase in susceptible maize line significantly reduced larvae growth in a bioassay using plant callus. The gene product of this proteinase was further shown to reduce caterpillar growth by disrupting the peritrophic matrix of the midgut (Pechan *et al*., 2002). A homologous cysteine proteinase to *mir1* from *Arabidopsis thaliana* (At4g11320) was also induced in response to either insect predation or MeJA treatment via the jasmonate-mediated signal transduction pathway (Reymond *et al*., 2004). It was also responsive to mechanical wounding, but not as strong as insect feeding and also, required the presence of an insect salivary factor for maximum expression.

Plants, like papaya, exude latex upon wounding, or during insect feeding. The papaya latex contains cysteine proteinases including papain, bromelain and ficin. Inclusion of these proteinases in an artificial diet at a concentration that occur in latex resulted in toxicity to silk worm larvae (Konno *et al*., 2003). In addition, the larvae died when fed on fig leaves, but not when the latex was removed by washing or when cysteine proteinases were inactivated by the inhibitor E-64. This shows that cysteine proteinases have a direct effect on larvae. The pro-region of papaya proteinase IV has also been found to inhibit digestive proteinases of the Colorado potato beetle (Visal *et al*., 1998). A secreted

papain-like cysteine proteinase of tomato, RCR3 (AAM19207), was required for the functioning of *Cf-2,* which is a resistance gene mediating recognition of the Avr2 avirulence gene of the fungal pathogen *Cladosporium fulvum resistance-2* (Krüger *et al*., 2002). However, the exact role of this apoplast localized RCR3 proteinase is still unclear.

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

Accession/Locus No.	*Gene Description*	*Stress type*	*Plant*	*Reference*
A. Induced				
At4g01620	*Cathepsin B-like cysteine proteinase*	*ABA, Drought*	*A. thaliana*	*Seki et al., 2002b*
At1g02300	*Cathepsin B-like cysteine proteinase*	*Drought and heat*	*A. thaliana*	*Rizhsky et al., 2004*
At3g19390	*Putative cysteine proteinase RD21A precursor*	*ABA*	*A. thaliana*	*Seki et al., 2002b*
At1g47128 (RD21A)	*Cysteine proteinase*	*Drought, ABA, Salt*	*A. thaliana*	*Seki et al., 2002a;2002b; Koizumi et al., 1993; Takahashi et al., 2004*
At4g39090 (rd19a)	*Thiol proteinase*	*Drought, Salt, ABA*	*A. thaliana*	*Koizumi et al., 1993; Hoth et al., 2002*
At4g16190	*Cysteine proteinase*	*ABA*	*A. thaliana*	*Hoth et al., 2002*
At4g16190	*Cysteine proteinase*	*Drought*	*A. thaliana*	*Seki et al., 2001*
At4g16190	*Cysteine proteinase*	*Phosphorus starvation*	*A. thaliana*	*Hammond et al., 2003*
At4g16190	*Cysteine proteinase*	*Sulfur deficiency*	*A. thaliana*	*Hirai et al., 2003; Nikiforova et al., 2003*
At4g16190	*Cysteine proteinase*	*Sucrose starvation*	*A. thaliana*	*Contento et al., 2004*
At5g60360	*Cysteine proteinase*	*Drought, Drought and heat*	*A. thaliana*	*Rizhsky et al., 2004*
At2g21430	*Cysteine proteinase*	*Drought*	*A. thaliana*	*Williams et al., 1994*
AK073373	*Papain like cysteine proteinase*	*Drought, high salt, ABA, cold*	*Oryza sativa*	*Rabbani et al., 2003*
Y10780	*Thiol proteinase*	*Desiccation*	*Sporobolus stapfianus*	*Blomstedt et al., 1998*
CD051336	*Cysteine proteinase*	*Dehydration*	*Cicer arientinum*	*Boominathan et al., 2004*
X99936 (SEE1)	*Cysteine proteinase*	*Chilling*	*Zea mays*	*Griffiths et al., 1997*
NTCP-23like	*Cysteine proteinase*	*Sulfur deficiency*	*Nicotiana tabacum*	*Wawrzynska et al., 2005*
AF019145 (mir1)	*33 kDa maize cysteine proteinase*	*Insect feeding, wounding*	*Zea mays*	*Pechan et al., 1999*
At4g11320	*Cysteine proteinase (similar to mir1)*	*Insect feeding, MeJA, wounding*	*A. thaliana*	*Reymond et al., 2004*
U32430	*Cysteine proteinase*	*BTH*	*Triticum aestivum*	*Görlach et al., 1996*
At5g60360	*Cysteine proteinase*	*Sucrose starvation*	*A. thaliana*	*Contento et al., 2004*
At3g19390	*Cysteine proteinase*	*Sucrose starvation*	*A. thaliana*	*Contento et al., 2004*
BCP-15	*Cysteine proteinase*	*Drought, low temperature*	*Brassica napus*	*Stroeher et al., 1997*
AJ250432 (CYP15a)	*Cysteine proteinase*	*Dehydration, Salt*	*Pisium sativum*	*Jones and Mullet, 1995*
At1g62710	*βVPE*	*Drought, Drought and heat*	*A. thaliana*	*Rizhsky et al., 2004*
At4g32940 &At2g25940	*αVPE and γVPE*	*Wounding*	*A. thaliana*	*Kinoshita et al., 1999*
AF172856 (TDI-65)	*Cysteine proteinase*	*Drought*	*Lycopersicon*	*Harrak et al.,*

			esculentum	*2001*
CAA57522	*Cathespsin B-like cysteine proteinase*	*Wounding*	*Nicotiana rustica*	*Lidgett et al., 1995*
AF055848	*Subtilisin-like proteinase*	*Mechanical wounding*	*A. thaliana*	*Cheong et al., 2002*
At4g21650	*Subtilisin-like proteinase*	*Drought, Drought and heat*	*A. thaliana*	*Rizhsky et al., 2004*
At1g62290	*Aspartic protease*	*Drought, Drought and heat*	*A. thaliana*	*Rizhsky et al., 2004*
At3g10410	*Putative serine carboxypeptidase*	*Drought and heat*	*A. thaliana*	*Rizhsky et al., 2004*
At5g47550	*Cysteine proteinase inhibitor*	*ABA*	*A. thaliana*	*Seki et al., 2002b*
At4g05110	*Cysteine proteinase inhibitor*	*Drought*	*A. thaliana*	*Seki et al., 2002a*
At4g05110	*Cysteine proteinase inhibitor*	*Drought and Heat*	*A. thaliana*	*Rizhsky et al., 2004*
At2g40880	*Cysteine proteinase inhibitor*	*Drought, cold*	*A. thaliana*	*Seki et al., 2001*
AF198390	*Tomato Multicystatin cystatin (TMC)*	*Wounding, MeJA, chitosan, OGA*	*Lycopersicon esculentum*	*Siqueira-Junior et al., 2002*
AF278573 (VuC1)	*Cowpea multicystatin*	*Drought*	*Vigna unguiculata*	*Diop et al., 2004*
U51854 (N2) & U51855 (R1)	*Inducible Cysteine proteinase inhibitors*	*Wounding, MeJA*	*Glycin max*	*Zhao et al., 1996*
CC8 (BN000515) & CC9 (BN000513)	*Corn cysteine proteinase inhibitors*	*Cold*	*Zea mays*	*Riccardi et al., 2004*
At5g12140 (AtCYS1)	*Cysteine proteinase inhibitor*	*Wounding, MeJA, Avirulent pathogen, NO*	*A. thaliana*	*Belenghi et al., 2003*
B24048	*Tomato Inhibitor II (Inh II)*	*Salt*	*Lycopersicon esculentum*	*Dombrowski et al., 2003*
AF330700 (SPLTI-a) AF404833 (SPLTI-b)	*Sweet potato proteinase inhibitor I gene family*	*Drought, chilling, ABA, MEJA*	*Ipomoea batatas*	*Wang et al., 2003*
BM378083	*Bowman-Birk trypsin inhibitor*	*Drought*	*Zea mays*	*Zinselmeier et al., 2002*
At2g43510	*Trypsin proteinase inhibitor*	*ABA*	*A. thaliana*	*Seki et al., 2002b*
At2g43510	*Putative trypsin inhibitor protein*	*Dehydration, manitol*	*A. thaliana*	*Takahashi et al., 2004*
At1g73260	*Trypsin proteinase inhibitor*	*ABA, drought*	*A. thaliana*	*Seki et al., 2002b; Hoth et al., 2002*
At2g43530	*Trypsin proteinase inhibitor*	*ABA*	*A. thaliana*	*Hoth et al., 2002*
At2g02120	*Protease inhibitor II*	*ABA*	*A. thaliana*	*Hoth et al., 2002*
AAB64325	*Putative trypsin inhibitor*	*Mechanical wounding*	*A. thaliana*	*Cheong et al., 2002*
At2g38870	*Putative protease inhibitor*	*Mechanical wounding*	*A. thaliana*	*Cheong et al., 2002*
AK064050	*Bowman-Birk trypsin inhibitor*	*Drought, high salinity, ABA*	*Oryza sativa*	*Rabbani et al., 2003*
AF039398	*Serine proteinase inhibitor II*	*Wounding, salt, ABA, electric current*	*Capsicum annum*	*Kim et al., 2001*
B. Down-regulated				
At4g16190	*Cysteine proteinase*	*Mechanical wounding*	*A. thaliana*	*Cheong et al., 2002*
	Senescence enhanced	*Mechanical*	*Nicotiana*	*Ueda et al., 2000*

	cysteine proteinase	*wounding*	*tabacum*	
At2g25940	*γ-VPE*	*High nitrate concentration*	*A. thaliana*	*Wang et al., 2000*
At2g34080	*Cysteine proteinase*	*Sucrose starvation*	*A. thaliana*	*Contento et al., 2004*
AC004401	*Serine carboxypeptidase*	*Mechanical wounding*	*A. thaliana*	*Cheong et al., 2002*
At4g30610	*Serine carboxypeptidase*	*ABA*	*A. thaliana*	*Hoth et al., 2002*
AC006929	*Serine carboxypeptidase*	*Mechanical wounding*	*A. thaliana*	*Cheong et al., 2002*
At1g15000	*Serine carboxypeptidase, putative*	*Sucrose starvation*	*A. thaliana*	*Contento et al., 2004*
At4g30020	*subtilisin-like serine protease*	*Sucrose starvation*	*A. thaliana*	*Contento et al., 2004*
At4g16500	*Cysteine proteinase inhibitor*	*ABA*	*A. thaliana*	*Hoth et al., 2002*
CCII (D38130), CC3 –CC6 (BN000508 - BN000511)	*Corn cystatins*	*Drought*	*Zea mays*	*Riccardi et al., 2004*

1.4.2 Plant proteinase inhibitors

Proteinaceous PIs are classified and named after classes of proteinases they inhibit that are serine, cysteine, aspartic and metallo-proteinases (Koiwa *et al*., 1997) (Table 1.3). Plant proteinaceous PIs are natural, defence-related proteins often present in seeds and tubers. They are induced in certain plant tissues/organs by herbivory or mechanical wounding (Ryan, 1990; Koiwa *et al*., 1997). PIs contribution to the plant defence mechanism relies the on inhibition of proteinases present in insect guts. PIs cause reduction in the availability of amino acids necessary for growth and development of insects or nematodes or they inhibit proteinases required for host-pathogen interaction. The activity of PIs depends on their capacity to form stable complexes with target proteinases, by blocking, altering, or preventing access to the enzyme active site.

Table 1.3 Families of plant proteinaceous proteinase inhibitors

Family	Proteinase Inhibited
Serine proteinase inhibitors	**Trypsin and chymotrypsin**
Soybean trypsin inhibitor (Kuntiz family)	
Bowman-Birk family	
Barley trypsin inhibitor family	
Potato inhibitor I family	
Potato inhibitor II family	
Squash inhibitor family	
Ragi I-2/ maize trypsin inhibitor family	
Serpin family	
Cysteine proteinase inhibitors	**Papain, cathepsin B, H, L**
Metallo-proteinase inhibitors	**Carboxypeptidase A, B**
Aspartic proteinas inhibitors	**Cathepsin D**

(Adapted from Koiwa *et al*., 1997).

1.4.2.1 Cysteine proteinase inhibitors

The cysteine proteinase inhibitors or cystatins constitute a superfamily of evolutionarily related proteins. They are reversible inhibitors of papain-like cysteine proteinases (Brown and Dziegielewska, 1997) and they have been identified in vertebrates, invertebrates and plants. Based on their sequence homologies, presence and position of disulfide bonds, and the molecular mass of the protein, the cystatin superfamily has been subdivided into three families (Turk and Bode, 1991; Sotiropoulou *et al*., 1997). Family 1 (stefins) comprises a single-chain protein lacking disulfide bonds and having a molecular mass of ~ 11 kDa. Family 2 (cystatins) is composed of a single chain protein of ~ 15 kDa and each family member has intra-molecular disulfide bonds. Family 3 (kininogens) are exclusively higher molecular weight glycoproteins with a molecular mass of ~ 60 – 120 kDa and they contain three family 1-like repeats.

The plant cystatins, also called phytocystatins (PhyCys), resemble family 1 as they lack a disulfide bond but have a higher amino acid similarity with family 2 cystatins. Abe *et al*.

(1991) proposed to place PhyCys into a separate 'phytocystatin' family. Recently, this has further been reconfirmed by the work of Margis *et al*. (1998). The authors showed that PhyCys contain a particular consensus motif [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N found in a region which corresponds to a predicted N-terminal α-helix and they cluster on a distinct branch separate from other cystatin families on the phylogenetic tree. In addition to this consensus, the PhyCys contain three motifs that are involved in the interaction with their target proteinases: (1) the active site motif QxVxG, (2) a G near the N-terminus and (3) a conserved W in the second half of the protein. Most PhyCys have a molecular mass in the range of 12–16 kDa. However, multicystatins of ~ 85 kDa from potato and ~ 87 kDa from tomato contain eight similar cystatin domains (Waldrom *et al*., 1993; Wu and Haard, 2000). In addition, cystatins from soybean (Misaka *et al*., 1996), cabbage (Lim *et al*., 1996), sesame (Shyu *et al*., 2004), barley, rice and Arabidopsis (Martinez *et al*., 2005) with a molecular mass of about 23 kDa containing an extended C-terminal end have also been described. This C-terminal extension is recognized as cystatin domains (http://www.sanger.ac.uk/Software/Pfam/). This was suggested to probably originate from a duplication event. However the cystatin motifs that interact with cysteine proteinases are not conserved in these C-terminal tails. This may indicate that theses regions have evolved to take a different role (Martinez *et al*., 2005). All the PhyCys described from Arabidopsis, rice and barley have a signal peptide with the exception of Hv-CPI and AtCYS-1. This indicates they are targeted to the endoplasmic reticulum (Martinez *et al*., 2005).

Since the discovery of the first PhyCys oryzacystatin I (OC-I) (Abe *et al*., 1987) nearly hundred cystatin sequences from over 30 different plant species have been identified/cloned. The detailed list of the inhibitor, their enzyme inhibitory activity and heterologous and transplant expression have been documented in the database for plant proteinase inhibitors (PLANT-PIs at http://bighost.area.ba.cnr.it/PLANT-PIs) (De Leo *et al*., 2002). PhyCys occur as a multigene family (Abe *et al*., 1987; Waldron *et al*., 1993) with varying degrees of structural and probably functional similarities among the members of the sub-families. However the endogenous role being played by each member of the family (whether similar or different) has still to be elucidated. So far, three cDNAs encoding soybean PhyCys (Botella *et al*., 1996), multigene PhyCys from sugarcane with 25 members (Reis and Margis, 2001) and 5 wheat PhyCys have been described (Corre-Menguy *et al*., 2002; Kuroda *et al*., 2001). Recently 8 further cystatins were described from a maize ESTs database (Massonneau *et al*., 2005). This increases the total number to 10 which also includes the two previously described CCI and CCII from maize kernels (Abe *et al*., 1992 and 1995). Database searches for the *Arabidopsis* and rice genome as well as for the barley EST databases have confirmed the presence of 12 cystatins in rice, 7 in *Arabidopsis*, 7 in barley (Martinez *et al*., 2005) including the cystatin AtCYS1 of *Arabidopsis* (Belnghi *et al*., 2003) and the barley cystatin Hv-CPI (Gaddour *et al*., 2001). Among the 12 rice cystatin members, a cystatin gene (OC-III) (Ohtsubo *et al*., 2005) has been found to have cathepsin B inhibitory activity. This is unlike OC-I and OC-II, which have more affinities towards papain and cathepsin H (Michaud *et al*., 1993a).

Despite the presence of cDNA sequences, functional characterization of individual cystatin members in a family and the possible regulation by biotic and abiotic factors are still to be addressed. Further limited information is currently available on cystatin expression and organ specificity in crops. Based on available information from studies on rice, corn and soybean cystatins, differential specificities against proteinases, and differential expression patterns in tissues/organs and in developmental stages are evident. This might imply their versatile role within the plant. Botella *et al*. (1996) showed differential regulation of three cystatins from soybean in organs of seedlings and plants. One of these was constitutively expressed and the other two were wound and MeJA-inducible in local and systemic leaves and they required ethylene for expression. Further, the wound or MeJA-inducible soybean PIs, N2 and R1, were more potent against insect digestive proteinases and they had higher papain inhibition activity than the constitutive homologue L1 (Zhao *et al*., 1996). It has been suggested that wound-inducible cystatins have a protective control against insect predation. In general, substantial variation exists among cystatins in activity and specificities against proteinases indicating a diverse range of targets.

1.4.2.2 Plant proteinase inhibitor expression under drought and heat stress

Unlike proteinases, regulation of plant PIs by abiotic stresses, other than mechanical wounding, has not been studied in great detail. The responses of endogenous plant PIs to abiotic stress have been investigated in only a few instances. Barley cystatin Hv-CP1 mRNA increased due to dark treatment and anaerobiosis and also after cold shock in vegetative tissues (Gaddour *et al*., 2001). In chestnut plants, cystatin RNA accumulated

in response to cold, saline or heat-shock in both leaves and roots (Pernas *et al*., 2000). In rice seedlings, exposure to the gaseous air pollutant SO_2 led to changes in a PhyCys-like protein (Rakwal *et al*., 2003).

Inhibitors belonging to the Kunitz-type BnD22 of *Brassica napus* (Downing *et al*., 1992) and WSCP of cauliflower (Nishio and Satoh, 1997) were induced by drought stress and salinity, respectively. Two sweet potato leaf trypsin inhibitors, which are constitutively expressed in unexpanded leaves, were induced by water deficiency, chilling, and osmoticant treatments, such as polyethylene glycol (PEG), sorbitol and NaCl, in mature fully expanded leaves. These stresses also enhanced inhibitor expression in unexpanded leaves (Wang *et al*., 2003). Further, they were wound, ABA and MeJA-inducible in locally damaged/treated leaves but lacked systemic expression. A *Capsicum annuum* serine proteinase inhibitor II (*CaPI-2*) expression was enhanced by mechanical wounding both locally and systemically and also in response to exogenous ABA, salt and electric current treatment while acetylsalicylic acid repressed wound-inducible expression of this PI (Kim *et al*., 2001). Localized heat treatment of tomato leaves also induced accumulation of protease inhibitor II in an ABA-deficient tomato mutant (Herde *et al*., 1996).

The developmental and preferential organ specific expression patterns of 10 maize cystatins were described by Massonneau *et al*. (2005). The authors also showed that drought stress represses expression of transcripts of 5 cystatins (CII, CC3, CC4, CC5 and CC9) in maize leaves, except CCI, which was not affected by drought stress. Two maize

cystatins (CC8 and CC9) were further induced by cold stress. This shows that not all cystatins are induced by abiotic stress. Salt stress resulted in accumulation of PIs and the activation of other wound-related genes in tomato (*Lycopersicon esculentum*) (Dombrowski, 2003). It was found that salt stress enhanced the plant response to wounding both locally and systemically, and the JA-dependent pathway was required for salt stress-induced accumulation of PIs. Prosystemin activity was not required but was necessary to achieve maximum level of PI accumulation (Dombrowski, 2003).

Two to three-fold variations in the level of wound-inducible PIs were reported among 8 varieties of *Capsicum annuum*. It was proposed that a wound response might be useful for genetic selection in enhancing the defence response of pepper plants to herbivores and pathogens (Moura and Ryan, 2001). The abundance of drought stress-inducible PhyCys accumulation has also been associated with tolerance to drought stress. Comparison of the proteome of drought-tolerant and susceptible maize inbred lines showed that a higher amount of cystatin was accumulated under drought stress when compared to non-stressed controls. Further, the level of accumulation of the cystatin was higher in a drought-tolerant inbred line. This was not related to the leaf water content and ABA accumulation and was rather related to genetic variation in protein regulation at the transcriptional, translational and post-translational level between the two lines (Riccardi *et al*., 2004). Similarly, a multicystatin VuC1 of cowpea (*Vigna unguiculata*) was induced in leaves of progressively drought-stressed cowpea plants. The level of transcript corresponded to the degree of tolerance or susceptibility of cultivars to drought stress, and the accumulation of the cystatin message was higher in tolerant cultivars (Diop *et al*., 2004). In contrast,

the level of induction of this multicystatin by other factors, such as ABA, was not as strong as drought stress.

Plant PIs that were up- or down-regulated under different stresses and the different treatments are shown in Table 1.2. A cystatin of *Arabidopsis* At2g40880 (AtCYS3) was induced by both drought and cold stress and this contains a 9 base pair conserved DRE in its promoter sequence (Seki *et al*., 2001). This element is an important *cis*-acting element in drought, high salt and cold-responsive gene expression in an ABA-independent manner (Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki and Yamaguchi-Shinozaki, 2000). Moreover, these authors have shown that this cystatin was a target of the transcription factor DREB1A (Seki *et al*, 2001). From a similar large-scale transcriptom study in *Arabidopsis* and rice (Seki *et al*., 2002a and 2002b; Hoth *et al*., 2002; Rabbani *et al*., 2003; Rizhsky *et al*., 2004) a number of PIs that are responsive to different stresses, ABA and MeJA are shown in Table 1.2. Some of the abiotic stress-inducible genes encoding PIs are likely to protect the proteins by inhibiting the activity of proteinases and some of which have been described as being also induced by abiotic stress (Ramanjulu and Bartels, 2002).

1.4.3 Plant engineering and the cysteine proteinase/proteinase inhibitor system

Losses of agricultural production due to pests and diseases have been estimated at 37% worldwide. In addition to crop damage caused by feeding insects, mites and nematodes cause additional yield losses by transmitting over 200 plant diseases (Haq *et al*., 2004). Traditional pest control involves the use of conventional pesticides, which in general are non-specific and wipe out other non-target insects, pollutes the agro-ecosystem and

increases the cost of production. An alternative strategy would be the enhancement of plant resistance to pests through integrated pest management (IPM) programs that comprises traditional cultural practices, judicious use of pesticide and exploitation of inherently resistant varieties that include genes encoding for anti-nutritional proteins. The use of transgenic crops expressing foreign insecticidal genes could significantly contribute to sustainable agriculture and could be an important component of IPM. In this regard, genetically engineered insect-resistant crop varieties, which express for example insecticidal proteins derived from *Bacillus thuringiensis* (Bt-toxins), have proven to provide an efficient way to control a number of major insect pests in crops like cotton and maize (for reviews see: Cannon, 2000; Hilder and Boulter, 1999; Peferoen, 1997). The reduced use of conventional pesticides on these genetically modified crops has lead, however, to an increased infestation by secondary pests (Cannon, 2000; Greene *et al*., 1999). Moreover, the constant presence of Bt-toxins in the crop plants and their acute toxicity to target insects creates a strong genetic selection for resistant phenotypes.

Cysteine PIs are one of the prime candidates with highly proven inhibitory activity against insect pests and certain pathogenic fungi, nematodes and viruses. Plants do naturally contain PhyCys at least in seeds and tubers and have been fed on by insects that have cysteine proteinases as digestive enzymes. This paradox was explained by the fact that naturally produced PhyCys do occur for example in rice seeds at a very low concentration of 0.001 – 0.002% (Kondo *et al*., 1989). This was thought to be insufficient for effective protection against insect pests having cysteine proteinases in excess of the PhyCys in the seeds eaten by the insects. Besides low level of expression, the

organ/tissue and developmental specificity of most PhyCys suggests that part of the plant or developmental stage could be susceptible to attack by insect pests requiring the need to enhance endogenous resistance of most crop plants by overexpression of PhyCys.

The effective use of PIs generally requires characterization and relative importance of proteinases used by the target pest, both for proteolysis and host plant-pathogen interaction. Studies have shown that the digestive proteolytic enzymes in the different orders of commercially important insect pests belong predominantly to one of the major classes of proteinases. Coleopteran and hemipteran species and parasitic nematodes tend to utilize cysteine proteinases for digestion of food proteins (Murdock *et al*., 1987; Ryan, 1990). The processing of a polyprotein by certain plant virus also requires cysteine proteinases, which can be targeted by PhyCys (Gutierrez-Campos *et al*., 1999). In contrast, lepidopteran insects mainly use serine proteinases (Ryan, 1990; Wolfson and Murdock, 1990).

The defensive role of PhyCys against insect predation has been based on the observation that PhyCys are induced by wounding and application of MeJA (Hildmann *et al*., 1992; Botella *et al*., 1996). Analysis of data from *in vitro* assays indicates that these proteins inhibit digestive cysteine proteinases in insect guts. Bioassays have shown that these proteins possess insecticidal activities against insects belonging to the coleopteran and hemipteran orders (Liang *et al*., 1991; Walsh and Srickland, 1993; Orr *et al*., 1994; Kuroda *et al*., 1996). Also, plants stably overexpressing cystatin cDNAs with enhanced resistance towards insects (Leplé *et al*., 1995; Lecardonnel *et al*., 1999: Delledonne *et al*.,

2001) nematodes (Vain *et al*., 1998; Atkinson *et al*., 2003), filed slug (Walker *et al*., 1999) and potyviruses (Gutierrez-Campos *et al*., 1999) were generated with promising results regarding protection against against pests and pathogenes. Transgenic plants expressing a cystatin transgene so far created to enhance resistance of plants against their target pests are listed in Table 1.4.

Exogenous PhyCys expression can be used to raise plants with a partial natural resistance to full resistance. This approach was shown in potato by expressing an exogenous PhyCys OC-IΔD86 (a modified OC-I) and also sunflower cystatin. Additional expression gave an additive effect in protection against plant parasitic nematodes by raising plants from partial resistance to full resistance (Urwin *et al*., 2003). It was also suggested that the *Mi* gene of tomato is effective against *Meloidogyne incognita* but not to the virulent populations of *M. javanica* or *M. hapla*. *Mi* resistance is temperature sensitive and breaks down at higher temperatures. PhyCys could widen the range of species for which resistance was effective in tomato and protect against the effects of high temperature and assist the durability of *Mi* (Urwin *et al*., 2003). PhyCys do offer several advantages over other PIs to be used for insect control. They do not harm non-target arthropods or perturb soil microorganism communities and they have been shown to be non-toxic to mammals (Cowgill *et al*., 2002a and 2002b; Ashouri *et al*., 2001; Atkinson *et al*., 2004). The existence of diversified forms of PhyCys in a wide range of plant species (De Leo *et al*., 2002) will ultimately allow selection of more effective PhyCys with potent activity against target pests. Further, with the technology and knowledge available in protein engineering, it will also be possible to increase the inhibition constant (K_i) of cystatin to

cysteine proteinases (Urwin *et al*., 1995) and also to be used with other PIs in gene pyramiding approaches (Outchkourov *et al*., 2004a).

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

Gene source	Transformed plant	Target pest/other purpose	Reference
OryzacystatinI (OC-I)	*Popular*	*Chrysomela tremulae* (Coleoptera: Chrysomelidae)	*Leplé et al.,1995*
	Potato	*Colorado potato beetle larvae (Leptinotarsa decemlineata)*	*Lecardonnel et al., 1999*
	Oilseed rape	*Cabbage seed weevile (Coleoptera: curculionidae)*	*Girard et al., 1998*
	Oilseed rape	*Myzus persicae*	*Rahbé et al, 2003*
	Tobacco	*Potyviruses*	*Gutierrez-Campos et al., 1999*
	Tobacco	*Physilogical studies under abiotic stresses*	*Van der vyver et al., 2003*
	Sweetpotato	*Seewtpoato feathery mottle virus*	*Cipriani et al., 2000*
Oryzacystatin IΔD86[1]	*Transgenic hairy roots*	*Globodera pallida*	*Urwin et al., 1995*
Oryzacystatin IΔD86	*Rice*	*Nematode resistane: Meloidogyne incognita*	*Vain et al., 1998*
		Rotylechulus reniformis	*Urwin et al., 2000*
Oryzacystatin IΔD86	*Arabidopsis thaliana*	*Field Slug: Derocerus reticulatum*	*Walker et al., 1999*
Oryzacystatin IΔD86	*Arabidopsis thaliana*	*Roor-knote nematode: Meloidogyne incognita* *Beet-cyst nematode: Heterodera schachtii*	*Urwin et al., 1997*
Oryzacystatin I and II	*Alfalfa*	*Root lesion nematode*	*Samac and Smigocki, 2003*
Sunflower cystatin and OCIΔD86	*Potato*	*Nematode: Globodera spp.*	*Urwin et al., 2003*
Corn Cystatin	*Rice*	*Maize grain weevil: Sitophilus zeamais*	*Irie et al., 1996*
Arabidopsis thaliana (AtCYS1)	*White popular (Populus albua)*	*Chrysomela: Chrysomela populi*	*Delledonne et al., 2001*
IΔD86 with root specific promoters	*Potato*	*Roor-knote nematode: Meloidogyne incognita* *Potato cyst nematode: Globodera pallida*	*Lilley et al., 2004*
Custom-made mulidomain protease inhibitor (K-A-C-P and EIM- K-A-C-P)[2]	*Potato*	*Western flower thrips Frankliella occidentalis (Thysanoptera: Thripidae)*	*Outchkourov et al., 2004a*

[1] *Oryzacystatin IΔD86: engineered OC-I.*

[2] Representative classes of inhibitors of cysteine and aspartic proteases (Kininogen domain [3](K), stefin A (A), cystatin C (C), potato cystatin (P) and/or equistatin (EIM) were fused into reading frames consisting of four or five proteins.

1.4.3 Stability of proteinase inhibitors transgenes in plants

Stable expression of a transgene in genetically engineered plants determines the success for adding a desired trait. Successful applications of proteinase inhibitors for pest control depend on the expression level of a selected inhibitor at concentrations of around 0.5-

1.5% of total soluble protein (Jongsma and Bolter, 1997). This can be achieved by targeting the protein into an appropriate sub-cellular environment to obtain proper folding and by protection of the inhibitor protein from unwanted degradation by plant proteinases. So far, the reasons for success or failure to express proteins in plants are not well understood. Some of the well known factors that can affect transgene expression in plants include transgene silencing due to a phenomenon called co-suppression. This arises when a copy of a gene is introduced into a plant resulting in silencing of both the introduced and the endogenous gene (Meyer and Saedler, 1996). The nature of the transgene itself can also affect the level of its expression. Good examples are the expression of the bovine spleen trypsin inhibitor in tobacco which varied about 20-fold depending on modifications of the cDNA at the 3'and 5' ends and by minor codon changes (Christeller *et al*., 2002). Similarly the A/U rich motif of wild-type Bt gene contributes to its mRNA instability after transcription and such sequence motif recognition can vary between plant species (De Rocher *et al*., 1998). Other problems like *in vivo* proteolysis by uncontrolled plant proteinases or degradation by post-translational ubiquitination, environmental abiotic stresses and possibly other epigenetic mechanisms can limit transgene expression and stability.

Abiotic stresses have been reported to influence transgene expression and stability and may determine the success of genetically modified crops under field conditions. Study on PI transgene expression and stability under abiotic stresses is very limited. The detrimental effects of environmental abiotic stresses on transgene expression and stability other than PI transgene have been documented in transgenic peas expressing α-amylase

inhibitor-1 (α -AI-1, Sousa-Majer *et al*., 2004) and in transgenic cotton expressing Bt-toxin (Olsen *et al*., 2001).

The expression of many traits in transgenic plants can be severely hampered by the individual characteristics of foreign genes and proteins not adapted to the specific subcellular environment of the new host. Proteolytic degradation of heterologous expressed proteins is still a limiting factor in the accumulation of many foreign proteins in plants (Dolja *et al*., 1998; Stevens *et al*., 2000; Outchkourov *et al*., 2003). Different approaches have been employed to increase accumulation of foreign proteins in plants. This includes changing the compartmentalization of the expressed proteins by targeting to and retention in the endoplasmic reticulum (Schouten *et al*., 1996) or directing the expression to chloroplasts (Wong *et al*., 1992). In different plant cellular compartments, a wide variety of proteinases are involved in the processing and degradation of proteins (Vierstra, 1993 and 1996). The levels of these plant proteinases are affected by many developmental factors such as seed maturation and germination, leaf senescence, combined with environmental biotic and abiotic stress (see above), that can limit expressed protein.

To obtain potato (*Solanum tuberosum* cv Desire´e) plants resistant to Colorado potato beetle (*Leptinotarsa decemlineata* Say) larvae, Outchkourov *et al*. (2003) expressed the proteinase inhibitor equistatin from sea anemone (A*ctinia equina*) under the control of a strong, light-inducible and constitutive promoter and targeted the inhibitor to the secretory pathway by adding an ER retention signal (KDEL). All constructs yielded protein degradation patterns, which considerably reduced the amount of active inhibitor

in planta. The authors identified arginine/lysine-specific and legumain-type Asn-specific cysteine proteinase that seriously impeded the functional accumulation of recombinant equitation *in planta* (Outchkourov *et al*., 2003). The degradation of equistatin was inhibited *in vitro* by Kininogen domain 3 while other cystatins including the potato cystatin prevented degradation to a lower degree. The authors have also expressed cystatin C, kininogen domain three, stefin A and equistatin in potato and found that except for stefin A the other proteinase inhibitors partially degraded *in planta.* Consequently, thrips feeding on plants expressing these inhibitors showed that plants expressing stefin A were the most deterrent to thrips (Outchkourov *et al*., 2004b). *In planta* degradation of cystatins was avoided by expressing the cystatins as custom-made multidomain inhibitor, in which representative classes of inhibitors of cysteine and aspartic proteases (Kininogen domain 3, stefin A, cystatin C, potato cystatin and/or equistatin) were fused into reading frames consisting of 4 or 5 inhibitors and joined by five glycine residues and transformed into potato (Outchkourov *et al*., 2004a).

CHAPTER TWO

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

2.1 Abstract

Expression of a rice cysteine proteinase inhibitor transgene, oryzacystatin-I (OC-I), was studied under drought, heat and a combination of both stresses in transformed tobacco plants (*Nicotiana tabacum* L. cv. Samsun). Transformed plants either exposed to individual or combined stresses had higher OC-I transcript and protein levels than non-stressed transformed plants and expressed OC-I was active against the plant cysteine proteinase papain. No OC-I degradation products could be detected in transformed plants by immuno-blotting following application of drought, heat or a combination of stresses. This indicates that OC-I transgene expression and the stability of the encoded protein in plants was not affected by drought or heat stress.

2.2 Introduction

The success of studying the function of an exogenous gene in a given plant background strongly depends on the expression of the gene and the stability of the expressed protein. Sub-optimal growth conditions might severely affect transgene expression and/or accumulation of encoded protein and activity. A study by Neumann *et al.* (1997) showed that heat treatment of transformed tobacco plants resulted in reversible reduction or complete loss of exogenous luciferase and neomycin phosphotransferase activity in 40% of transformed tobacco plants. Similarly, Sousa-Majer *et al*. (2004) found that the level of α-amylase inhibitor 1 (α-AI-1) in transgenic peas (*Pisum sativum* L.) was reduced both in the amount and protective ability against a seed weevil as a result of exposure to high temperature (32/27°C day/night). In addition, plant endogenous proteinases (Outchkourov *et al*., 2003), developmental stages of plants as well as environmental abiotic stresses that enhance abundance of certain plant endogenous proteinases can be a limiting factor in the accumulation of foreign proteins in plants (Dolja *et al*., 1998; Stevens *et al*., 2000; Down *et al*., 2001). However, the stability and expression of an exogenous cystatin expressed in a transformed plant during stress has so far not been studied in detail.

The aim of this part of the study was to investigate the expression and the stability of the OC-I transgene under drought and heat stress and a combination of these stresses. For that, different molecular and biochemical methods were used to study (i) the integration of the transgene into the plant genome, such as PCR and Southern blotting, (ii) to analyse the expression level of the gene by using northern blotting for transcript detection, and

(iii) to detect protein expression by either immuno-blotting or enzymatic assays to monitor protein activity.

2.3 Materials and Methods

2.3.1 Plant material

Transformed plants (T4/5) of the cultivar 'Samsun' (Tobacco and Cotton Research Institute at Rustenburg/South Africa) were produced following the procedure outlined by Horsch *et al*. (1985). They carried a *gus* marker gene coding for β-glucuronidase (GUS) and the gene coding for OCI under the control of a constitutive cauliflower mosaic virus (CaMV) 35S promoter (Van der Vyver *et al*., 2003). The non-transformed plants used this experiment and in other subsequent experiments refer to plants that passed through an identical transformation process and that have been selected from segregating population of primary transformation event. These plants lacked the insertion of the genes coding for GUS and OCI.

2.3.2 Detection of OC-I sequence in transformed plants

Genomic DNA was isolated from putative young leaves of transformed and control tobacco plants using a commercial DNA isolation kit (Amersham Phyto Pure DNA isolation kit, Amersham, UK). The quantity and quality of genomic DNA was determined by the NanoDrop® reading technique. A standard Polymerase Chain Reaction (PCR) using 100 ng genomic DNA as template was applied to amplify a portion of the OC-I coding sequence from transformed tobacco plants with a OC-I forward (5'-TCACCGAGCACAACAAGA-3') and reverse (5'– CATCGACAGGCTTGAACT - 3') primer. Plant DNAs, from which DNA bands of the expected size of 200 base pairs (bp) could be amplified and visualized on a 1.5% agarose gel, were considered as transformed with OC-I. These plants were labelled and transplanted to 5 l capacity pots containing 1:1

river sand/coconut coir potting media along with a corresponding non-transformed control plant and used for induction of drought, heat and combination of drought and heat stress two weeks after transplanting.

2.3.3 Stress treatment of plants

The experiment was conducted at growth cabinate facility situated on experimental farm of the University of Pretoria, South Africa. The two types of tobacco (Samsun) plants, transformed and non-transformed (non-transformed plants used in all experiments described in this thesis are obtained from segregating population of primary transformants after twice selfing) were treated with two growth temperatures consisting of treatments at: 26/20 ± 2°C (normal temperature) and 38/30 ± 2°C day/night (heat treatment) at 12 hours light photoperiod, and also two water regimes consisting of treatments with 25 – 35% (drought stress) and 80 – 100% field capacity (non-drought stress). This experimental design resulted in a 2 x 2 x 2 factorial treatment combination in a randomised complete block design, where each treatment set was replicated ten-times. Drought stress treatment was induced based on a gravimetric method. Watering was done on a daily basis by weighing individual pots based on the field capacity determination and the treatments. Light in the growth cabinet was provided by a combination of incandescent and fluorescent lamps generating a photosynthetic photon flux density of 240 ± 10 µmol m^{-2} s^{-1}. The relative humidity in the individual cabinet during the study period was 60 ± 4%. Plants received Hoagland nutrient solution three-times a week. Four weeks after treatment induction, leaf samples were collected from fully expanded 3rd or 4th leaf position from shoot tip, flash frozen in liquid nitrogen and either immediately

used or stored at -80°C until needed. The entire experiment was repeated twice. Leaf samples from both experiments were used as DNA and RNA sources in the analysis.

2.3.4 Preparation of leaf protein extract

Frozen leaf samples were homogenized in liquid nitrogen in the presence of an extraction buffer. The buffer contained 50 mM Tris-HCl, (pH 8), and to block proteinase activity, 1 mM PMSF (phenylmethylsulphonyl fluoride), 1 mM EDTA (Ethylenediaminetetraacetic acid), 10 µM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64) and 10 µM pepstatin A. The homogenate was centrifuged at 13000 rpm at 4°C for ten minutes in an Eppendorf centrifuge (Eppendorf, Gemany) and the resulting supernatant was used for further analysis after determination of the protein concentration according to Bradford (1976) using BSA as a standard (Bio-Rad, Hercules, USA).

2.3.5 Immuno-blotting

Protein containing supernatants from leaf homogenates were added to an equal volume of a 2X sample-loading buffer (90 mM Tris-HCl, pH 6.8; 20% glycerol; 2% SDS, 5% (v/v) β-mercaptoethanol and 0.2% bromophenol blue) and boiled at 93°C for 4 minutes. Boiled protein extracts were subjected to 12% (w/v) SDS-PAGE according to Laemmli (1970). Separated proteins on gels were then transferred to a Hybond™ P membrane (Amersham, UK), and blocked overnight at room temperature with gently shaking in a solution of 5% (w/v) low fat milk powder in Tris-buffered saline (TBS-T) containing 0.1% Tween-20. Blots were incubated for 1 hour under gentle shaking in primary OC-I antiserum (for detection of OC-I) or alternatively in primary Rubisco antiserum (for detection of the Rubisco small and large subunits). Antisera were diluted 1:5000 in TBS-T for 1 hour and

blots were washed three-times each in TBS-T containing 0.5% low fat milk. This was followed by incubation in horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, UK) allowing fluorescence detection. Anti-rabbit IgG was used as the secondary antibody (1:10000 dilution). Treatment was done under gentle shaking for 1 hour at room temperature followed by three washes of blots for 10 minutes each in TBS-T containing 0.5% low fat milk. Detection of labelled proteins was done by chemiluminescence using an ECL™-Plus kit (Amersham, UK) according to the instructions by manufacturer.

2.3.6 Cysteine proteinase inhibition by tobacco leaf protein extract

A protein extract was prepared as outlined under immuno-blotting, but without addition of any proteinase inhibitors. The supernatant was used after centrifugation for an papain inhibition assay after protein quantification. A stock solution of papain dissolved in 0.1 M citrate phosphate buffer (pH 6.0) was diluted to 2 µg ml $^{-1}$ protein in proteolysis buffer (0.1 M citrate phosphate buffer, pH 6.0, containing 10 mM L-cysteine). The papain solution (100 µl) was pre-incubated with or without 100 µg leaf extract from either transformed or non-transformed tobacco plants in a total reaction volume of 300 µl. After 10 minutes of pre-incubation at 37°C, 200 µl of the cysteine proteinase substrate benzyloxycarbonyl-phenylalanine-arginie aminomythylcoumarin (20 µM Z-phe-arg-AMC), prepared by diluting in proteolysis buffer of a 1.0 mM in dimethylsulfoxide stock) was added to the reaction mixture and the resulting mixture was incubated again for another 10 minutes at 37°C. The reaction was then stopped by addition of 1.0 ml stopping reagent to the reaction mixture. The stopping reagent contained 10 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid, pH 4.3. The fluorescence of the released AMC was determined by using a fluorescence

spectrophotometer (Hitachi Model F-2000) at an excitation and emission wavelengths set at 370 nm and 460 nm, respectively.

2.3.7 Southern blot analysis

Genomic DNA (20 µg) from transformed and non-transformed tobacco plants was digested overnight with restriction enzymes *Bam*HI, *Eco*RI, *Kpn*I, *Xba*I. Digested DNA was then run on 1% agarose gel and then transferred to a Hybond™-N+ membrane (Amersham, UK) according to the protocol described by Sambrook *et al*. (1989). A *Pst*I/ *Eco*RI cut of the OC-I coding sequences from plasmid pBluescript SKII and labelled by Random-Prime Labelling (Amersham, UK) was used as a probe. Hybridisation of blotted DNA with the probe was performed overnight after pre-hybridisation for 1 hour at 60°C in a hybridisation buffer (5X SSC, 0.1% SDS, 5% w/v dextran sulphate and 20-times dilution of liquid block, supplied with CDP- Star™ detection kit). Subsequent stringency washes and the detection with Gene Images™ CDP- Star™ and exposure to Hyper™ film were performed according to the manufacturer's instruction (Amersham, UK).

2.3.8 Northern blot analysis

Total RNA from transformed and non-transformed tobacco plants was extracted using the TriPure total RNA isolation kit (Roche, Germany). Quality of RNA was tested after running the RNA on a denaturing agarose gel and staining the RNA with ethidium bromide. RNA was quantified using the NanoDrop® technique. Northern blot analysis was carried out essentially as described by Sambrook and Russell (2001) with minor changes. Total RNA (20 µg) was first size-separated on a 1.2% agarose gel containing 2.2

M formaldehyde and then transferred to a Hybond-N+ membrane (Amersham, UK) and the DNA was UV cross-linked. For detection of an OC-I transcript, a 200 bp PCR amplified product representing the OC-I coding region was used as a probe after labelling with Random-Prime (Amersham, UK).

For the PCR reaction, the plasmid pBluescript SKII containing the cloned OC-I insert was used as a DNA template. For obtaining a probe for the Rubisco small subunit (*rcbS*), a portion of its coding sequence (359 bp) was amplified by PCR using designed primers from the tobacco *rbcS* cDNA sequence available on public database (GenBank accession AY220079). The primers used were forward 5-GCTGCCTCATTCCCTGTTTTC-3' and reverse 5'-TATGCCTTCTTCGCCTCTCC-3'. Both OC-I and *rbcS* probes were labelled by a Random-Prime labelling kit according to the manufacturer's instruction (Amersham, UK). Pre-hybridization for 2 hours and hybridisation of the probe with a membrane bound RNA were performed overnight at 65°C in a hybridisation buffer containing 0.5 M Na_2HPO_4, pH 7.2, 7% (w/v) SDS and 1mM EDTA. Subsequent stringency washes were performed at 65°C for 15 minutes each. The first washing solution contained 0.1% SDS (w/v), 2X SSC, the second solution 0.1% SDS (w/v), 1X SSC and the third washing solution 0.5X SSC and 0.1% SDS. Detection with the Gene Images™ CDP- Star™ and exposure to the Hyper™ film were performed according to the manufacturer's instruction (Amersham, UK).

2.4 Results

2.4.1 OC-I gene and protein detection

When different putative transformed tobacco plants expressing GUS were tested for the OC-I gene insertion by PCR analysis, all putative transformed plants showed an amplified PCR fragment with the predicted size of about 200 bp (Figure 1A; lanes T1-T4). An identical fragment was not amplified from genomic DNA obtained from a non-transformed plant (Figure 1A; lane NT). To test for the presence of an OC-I transgene encoded protein, an immuno-blot analysis was carried out using a polyclonal antibody raised against OC-I. Figure 1B shows the predicted OC-I protein band with an approximate size of 11.5 kDa detected from four putative transformed plants (Figure 1B; lanes T1-T4). A similar protein band was not detected in an extract from a non-transformed plant (Figure 1B; lane NT).

After restriction enzyme digestion of genomic DNA obtained from putative transformed plants, several hybridization products were detected when probed with an OC-I coding sequence. When *Bam*HI was used, three bands were detected (Figure 1C) and two bands were detected after digestion with either restriction enzymes *Eco*RI, *Kpn*I or *Xba*I. Such hybridization profile, however, was not detected in *Bam*HI digested genomic DNA obtained from a non-transformed tobacco plant (Fig 1C; lane NT). For all restriction enzymes used, only one major band beside less intense bands could be detected. This possibly indicates a single gene integration of exogenous OC-I into the tobacco genome and any detected additional band might have originated from cross-hybridization with endogenous tobacco cystatin sequences.

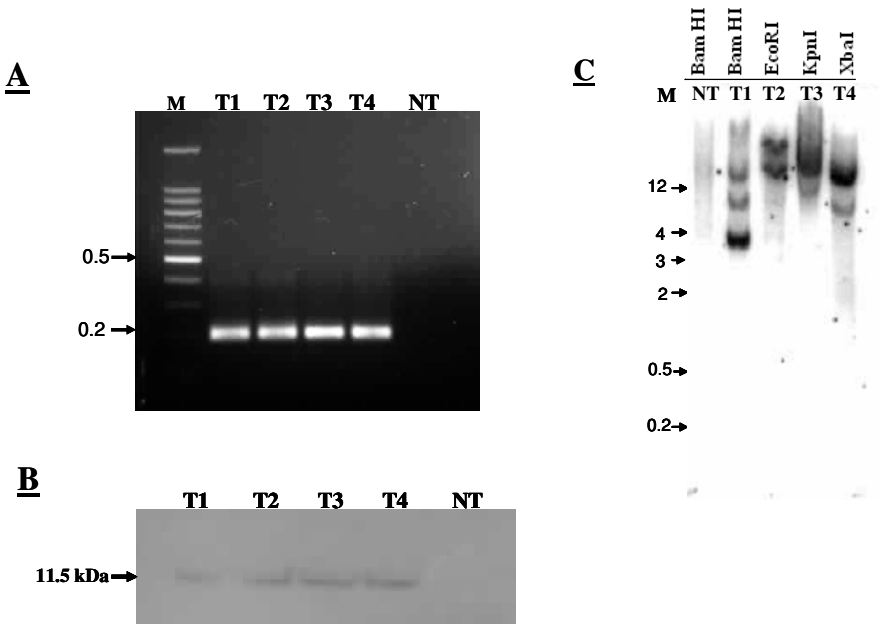


Figure 2.1 Characterization of putative transformed plants. (A) PCR amplified genomic DNA extracted from different putative transformed plants (lanes T1 –T4) and a non-transformed tobacco plant without OC-I gene insert (NT) as a control. M represents a 0.1 kbp DNA size marker (Roche, Germany). (B) Immuno-blotting of leaf protein extracts from different transformed plants (lanes T1-T4) and a non-transformed plant (lane NT) detected with an antibody raised against OC-I. (C) Genomic Southern blot analysis of genomic DNA derived from different transformed tobacco plants (lanes T1 – T4) and from a non-transformed tobacco plant (NT) probed with the complete OC-I sequence (598 bp) after digestion of genomic DNA with different restriction enzymes and transfer of the DNA onto a Hybond-N+ membrane. M represents a 1 kbp DNA size marker (Invitrogene, USA).

2.4.2 Expression of the small and large subunit of Rubisco

Transcription level of the small subunit (SSU) of the Rubisco gene (*rbcS* gene) and expression of the Rubisco large subunit (LSU) and SSU were used as indicators for the action of different stresses on transformed and non-transformed tobacco plants. On the protein level, LSU and SSU content were reduced in both transformed and non-transformed plants under drought stress when compared to non-stressed controls (Figure A). This was in comparison to heat-stressed or plants stressed with a combination of both stresses. On the transcription level, only transcription of SSU for non-transformed plants was greatly reduced under drought stress (Figure B). With all other stress treatments no obvious reduction in transcription was found when compared to transcription of SSU in a control plant.

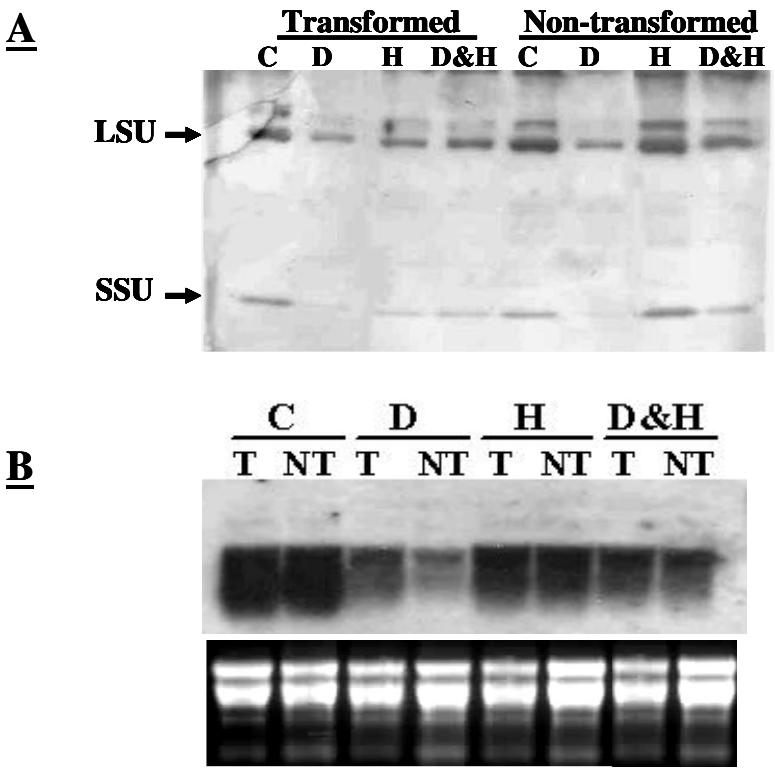


Figure 2.2 (A) Immuno-blot analysis of the a large subunit (LSU) and small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in transformed (T) and non-transformed (NT) tobacco plants under non-stress (C), drought (D), heat (H) and combined drought and heat stress (D&H). Polyclonal antibody for Rubisco subunit detection was raised against barley (*Hordieum vulgarae*) Rubisco (kindly provided by Dr Kepova K. Bulgarain Academy of Sceinces). (B; upper part) Northern blot analysis of the transcript of the small subunit of Rubisco (*rbcS* gene) and ethidium bromide stained total RNA (lower part) to ensure equal loading of RNA.

2.4.3 OC-I expression under stress

When protein extracts from transformed plants exposed to drought, heat and a combination of both stresses were analyzed by immuno-blotting using an OC-I antiserum, expression of exogenous OC-I could be detected in both non-stressed and stressed plants. A protein band of approximate 11.5 kDa was found (Figure 3A). The level of expressed OC-I increased by about 65% under both drought and heat stress and by 100% under a combination of drought and heat stress when compared to non-stressed transformed plants. Figure 4B shows the increase of the transcript level of OC-I following exposure of plants to the different types of stresses when compared to non-stressed transformed plants. All stress treatments increased transcription of OC-I in transformed plants.

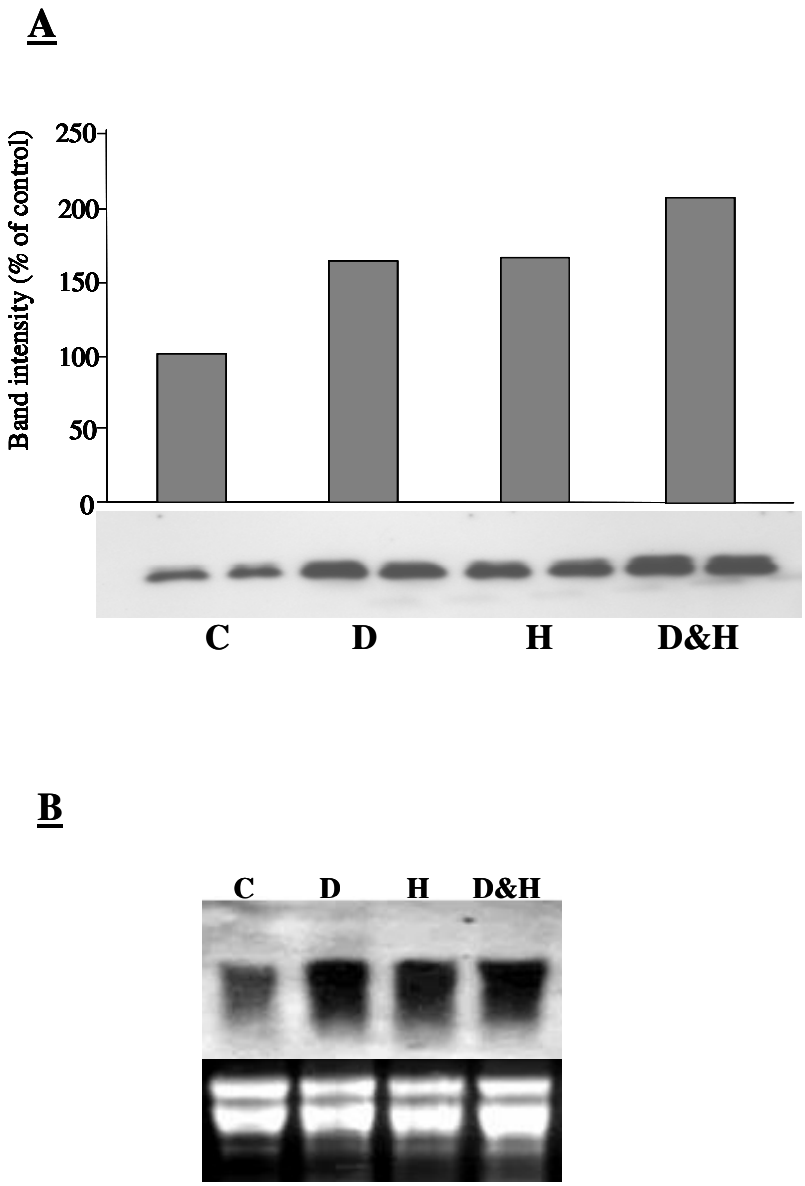


Figure 2.3 (A) Immuno-blot analysis to detect expression of OC-I in transformed non-stressed tobacco (C) and in transformed tobacco plants exposed to drought (D), heat (H) and a combination of drought and heat stress (D&H). Bars indicate average protein band intensity relative to the band from a non-stressed transformed plant. (B; upper part) Northern blot analysis for detection of an OC-I transcript in stressed and non-stressed

tobacco plants and B lower part ethidium bromide stained total RNA to ensure equal loading of RNA.

2.4.4 Activity of expressed OC-I

In order to test the activity of expressed exogenous OC-I, an *in vitro* cysteine proteinase activity assay was applied using a plant cysteine proteinase, papain, and the synthetic cysteine proteinase substrate Z-Phe-Arg-AMC. This substrate releases fluorescent AMC upon the action of a cysteine proteinase which can be quantified using fluorescence spectrophotometer. Figure 4A shows the residual activity of papain after incubation with a plant protein extract obtained from either non-stressed or stressed transformed or non-transformed plants. Inhibition of papain by a leaf protein extract from stressed transformed plants was greater than for an extract from non-transformed plants. Inhibition from extracts of non-transformed plants was 17% under drought, 13% under heat and 20% under both drought and heat stress when compared to 5% obtained from a non-transformed, non-stressed plant extract. In contrast, inhibition of papain from a non-stressed transformed plant extract was 24% and the level of inhibition was 45% for an extract derived from drought treated plants, 41% for heat and 54% for a combination of both stresses (Fig 4A). Inhibition was therefore always greater ($P<0.05$) for extracts from transformed plants than for extracts from non-transformed plants. E-64, a known inhibitor of cysteine proteinases, almost totally inhibited activity of papain (98%) under this experimental condition.

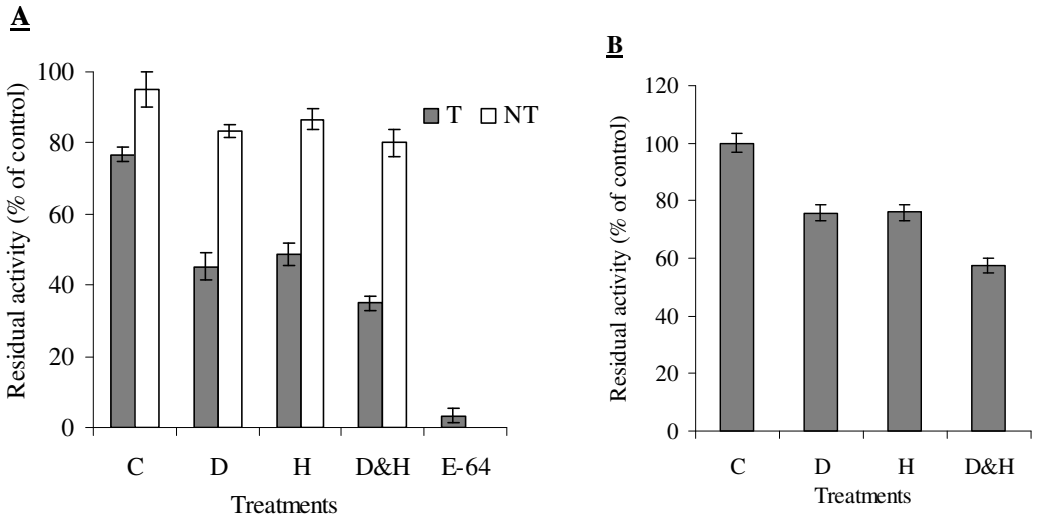


Figure 2.4 Residual papain activity (%) in the presence of 100 µg of plant soluble proteins. (A) Proteins derived from non-transformed (NT) and from OC-I expressing transformed (T) tobacco plants either un-stressed (C) or exposed to drought (D), heat (H) or a combination of both stresses (D&H). (B) Residual papain activity (%) after addition of a leaf protein extract derived from non-stressed transformed plants (C) and transformed plants stressed with drought (D), heat (H) and a combination of drought and heat (D&H). Values shown in A and B are relative to a papain activity without addition of a plant extract. Bars represent the mean of 4 different experiments ±SE.

2.5 Discussion

This part of the study showed that, the expression of the OC-I transgene in tobacco measured on the transcript and translational level was not reduced by drought, heat or a combination of both stresses when compared to non-stressed tobacco plants. Even further, an increase in the amount of both OC-I transcript and protein of OC-I was found. So far there is no evidence that the 35S CaMV promoter sequence, which is controlled in its activity by tissue and plant developmental stages (Benfey *et al.*, 1989), provides higher expression activity under drought or heat stress. Therefore, since equal amounts of transcripts and proteins were analyzed in the experiments from stressed and non-stressed plants, the increased transcript and protein level could be due to maintenance of an unchanged mRNA and protein level, while the majority of other transcripts decreased following stress treatment. In contrast, when an equal amount of RNA or protein was used for comparison to detect the Rubisco SSU and LSU protein or a SSU transcript, drought stress significantly reduced the amount of both protein and RNA transcripts.

Degradation of an exogenous protein can occur naturally or under stress. Expressed corn cystatin in transgenic rice showed a slight natural degradation in leaves (Irie *et al*., 1996). Further, Outchkourov *et al*. (2003) also found stepwise degradation of potato cystatin (PhyCys), equistatin from Sea Anemone and a chicken egg white cystatin in transgenic potato plants. This was very likely caused as a result of action of certain sub-groups of plant endogenous cysteine proteinases. Such degradation following heat stress has also been found with other expressed exogenous proteins. Further, heat stress has shown to promote degradation of exogenous proteins in transgenic plants. Peas expressing a seed

specific bean α -amylase inhibitor 1 resulted in reduction of both the quantity and activity of the transgene encoded protein (Sousa-Majer *et al*., 2004). Similarly, loss of transgene-encoded activity that directly correlated with a stressful heat treatment was observed in a single-cell-suspension culture of alfalfa *(Medicago sativa)* carrying a single copy of an introduced synthetic phosphinothricin-resistance gene (Eckes *et al*., 1989 and Walter *et al*., 1992). Neumann *et al*. (1997) further showed that heat treatment of transformed tobacco lines can result in reversible reduction or complete loss of exogenous luciferase and neomycin phosphotransferase activity in transformed tobacco plants. Any degradation of OC-I was not evident in this study despite exposing plants to drought or heat stress because none of the immuno-blotting experiments showed any OC-I degradation products.

In the following chapter the consequences of a stable expressed OC-I under stressful conditions in a transgenic plant have been investigated in greater detail. A major focus was on the investigation of any benefit for the plant under drought and heat stress by measuring general growth parameters and photosynthetic activity.

CHAPTER THREE

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

3.1 Abstract

Growth characteristics of transformed tobacco (Nicotiana tabacum L. cv Samsun) plants expressing a rice cystatin (OC-I) gene and non-transformed tobacco plants in response to drought, heat and a combination of theses stresses were measured. For the experiments two water regimes, which was well watered (80 – 100% field capacity) and drought stress (25 – 35 % field capacity) and two temperature regimes were used which was a 26/18ºC day/night cycle and a 38/30ºC day/night cycle. Measurement of plant growth characteristics showed that individual stresses significantly reduced plant growth and net photosynthetic rates of both transformed and non-transformed plants as compared to non-stressed tobacco plants. The degree of reduction of both parameters was further greater in plants challenged with a combination of drought and heat stress. Although under non-stress condition non-transformed plants had slightly higher total dry mass, photosynthetic rates and plant height, no highly significant differences could be found between the two types of plants in their response to a drought or heat stress or a combination of the stresses. Over-expression of exogenous OC-I in transformed tobacco did not confer any increased tolerance to drought or heat stress.

3.2 Introduction

About one third of the world's arable land suffers from inadequate supplies of water for agriculture, and in virtually all agricultural regions, yields of rain-fed crops are periodically reduced by drought (Kramer 1980; Boyer1982). Drought and heat stress almost invariably co-occur under arid-region field conditions and limit crop productivity. Drought stress hampers productivity by reducing or modifying the plant's essential processes like photosynthesis (Chaves *et al*., 2002; Lawlor, 2002; Lawlor and Cornic, 2002), which is reflected in decreased growth and productivity at the whole plant level. Similarly, temperature extremes above the optimal requirement for a plant can limit plant growth and productivity by impairing plant function including photosynthesis (Law and Crafts-Brandner, 1999) and reproductive development (Prasad *et al*., 2002). Drought stress can also cause oxidative damage to plant molecules like enzymes, lipids, RNA as a result of imbalance between production of reactive oxygen species and their metabolism (Foyer *et al*., 1994; Noctor *et al*., 2000). Combined drought and heat stress has been further shown to limit crop productivity more than the individual stresses (Craufurd and Peacock, 1993; Jiang and Huang, 2001). At the molecular level, the plant response to combined stress was also found to be greater than for individual stresses (Rizhsky *et al*., 2004). Further varietal/cultivar differences within a species in response to these stresses have been identified (Jagtab *et al*., 1998; Xu and Huang, 2001; Solomon and Labuschagne, 2003).

The aim of this part of the study was to investigate plant growth characteristics of transformed tobacco plants expressing an exogenous OC-I gene under drought and heat

stress. In particular, the plant growth and photosynthetic activity of OC-I expressing transformed tobacco plants were determined and compared to non-transformed tobacco plants. Plant growth characteristics measured included total plant dry mass, leaf area, leaf number and plant height. Leaf gas leaf gas exchange was measured to determine photosynthetic activity. The result obtained showed only marginal differences in plant growth and photosynthetic activity between transformed and non-transformed tobacco plants in response to drought or heat stress or a combination of the stresses.

3.3 Materials and Methods

3.3.1 Glasshouse experiments

Seeds from OC-I expressing transformed and non-transformed tobacco plants that have been selfed twice were raised on germinating tray on a glasshouse maintained at 26/20ºC day/night temperatures and 12 hours light with photosynthesis photon flux density of 240 ± 20 µmolm^{-2} s^{-1} and a relative humidity of 60 ± 4%. Four weeks seedlings of transformed and non-transformed seedlings were then transplanted to 5 litre capacity plastic pots filled with sand and coconut coir (50:50 by volume) and transferred to a glasshouse and well watered for two weeks. The growth condition was in the glasshouse during the study period were a relative humidity in the range of 40 – 80%, a temperature 26 ± 4ºC and a photosynthesis photon flux density of 650 - 900 µmol m^{-2} s^{-1}.

Drought stress was induced based on a gravimetric method. For that, half of the experimental plants were left without watering until plants showed wilting symptoms (10 days) while the remaining half were maintained at 80 – 100% field capacity. The amount of water evaporated was monitored daily by weighing unplanted pots placed randomly between planted pots in both stressed and non-stress treatments in each block. Pots were watered with the amount of water equivalent to the loss of weight. This was done to bring them to the pre-determined level of moisture whenever the weight of pots fell below the lower limit established for the treatment (25 – 35% for drought and 80 – 100% for non-drought stress treatments) until the end of the experiment. Plants received Hoagland nutrient solution 3-times a week. Two weeks after drought stress, the rate of photosynthesis was measured using a portable photosynthesis system (CIRAS-1, 1998, UK) on 10

randomly selected plants from each treatment combination. The rate of photosynthesis was measured on the 3rd or 4th fully expanded younger leaf counted from the shoot apex. The photosynthetic photon flux density incident at the level of the leaf was in the range of 770 - 850 µmol m^{-2} s^{-1}. Plants from 32 replicates per treatment were harvested three weeks after drought stress treatment. At harvest, plants were separated into leaves, stems and roots. Roots were washed off any soil debris. The leaf area was measured using a leaf area meter (Li-3000A, LI-COR, Inc. Lincoln, USA). The dry weight of roots and shoots was determined after drying the plant material at 70°C to a constant weight.

3.3.2 Growth cabinet experiments

Seedlings were raised as outlined above and eight weeks old seedlings were used for this experiment. The experiment with transformed and non-transformed plants included treatment with two growth temperatures, which were 26/20 ± 2°C and 38/30 ± 2°C, and two water regimes which were 25 – 35% and 80 – 100% field capacity. This resulted in a 2 x 2 x 2 factorial treatment combination in a randomized complete block design, where each treatment set is replicated 10-times. Drought stress treatment was induced based on a gravimetric method and watering was done as outlined above. Light in the growth cabinet was provided by a combination of incandescent and fluorescent lamps generating a photosynthetic photon flux density of 240 ± 10 µmol m^{-2} s^{-1}. The relative humidity in the growth cabinet during the study period was 60 ± 4%. Plants received Hoagland nutrient solution 3-times a week. Net photosynthesis was measured (as in experiment outlined above) four weeks after stress treatment. For measurements, plants were moved out of the growth cabinet. Plants were harvested six weeks after stress treatment

induction for growth characteristic measurements and data were collected as outlined in glasshouse experiment above.

3.3.3 Statistical analysis

Plant total dry mass, leaf area, leaf number, plant height, root/shoot ratios and net photosynthesis were analyzed as two factor experiment (genotypes, drought treatment) in the glasshouse experiment and as three factor (genotypes, drought and heat treatments) in growth cabinet experiment. ANOVA model of Minitab release 11.12 software package Minitab Inc (1996) was used for statistical analysis. Differences were considered significant at $P \leq 0.05$.

3.4 Results

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

A significant difference ($P<0.01$) in total dry mass, leaf area, leaf number, plant height and shoot/root ratio was found when drought stressed and non-stressed plants were compared. Drought stress reduced total dry mass by 58%, leaf area by 58%, plant height by 50% and leaf number by 37% in both transformed and non-transformed plants (Figure 1A-D). In contrast, drought stress increased the root/shoot ratio by 40% in plants when compared to non-stressed plants (Figure 1E). Although non-transformed plants were slightly higher and had greater total dry mass and net photosynthetic rates (10-15%) when compared to transformed plants once these differences were not highly significant ($P>0.05$).

When leaf gas exchange was measured, a significant difference ($P<0.01$) was found between drought stressed and non-stressed plants (Tab. 1). Net photosynthetic rate was reduced by 75%, stomatal conductance by 84% and transpiration rate by 77% in both transformed and non-transformed plants. However, no significant difference was found between transformed and non-transformed plants in net photosynthetic rate, stomatal conductance and transpiration rates.

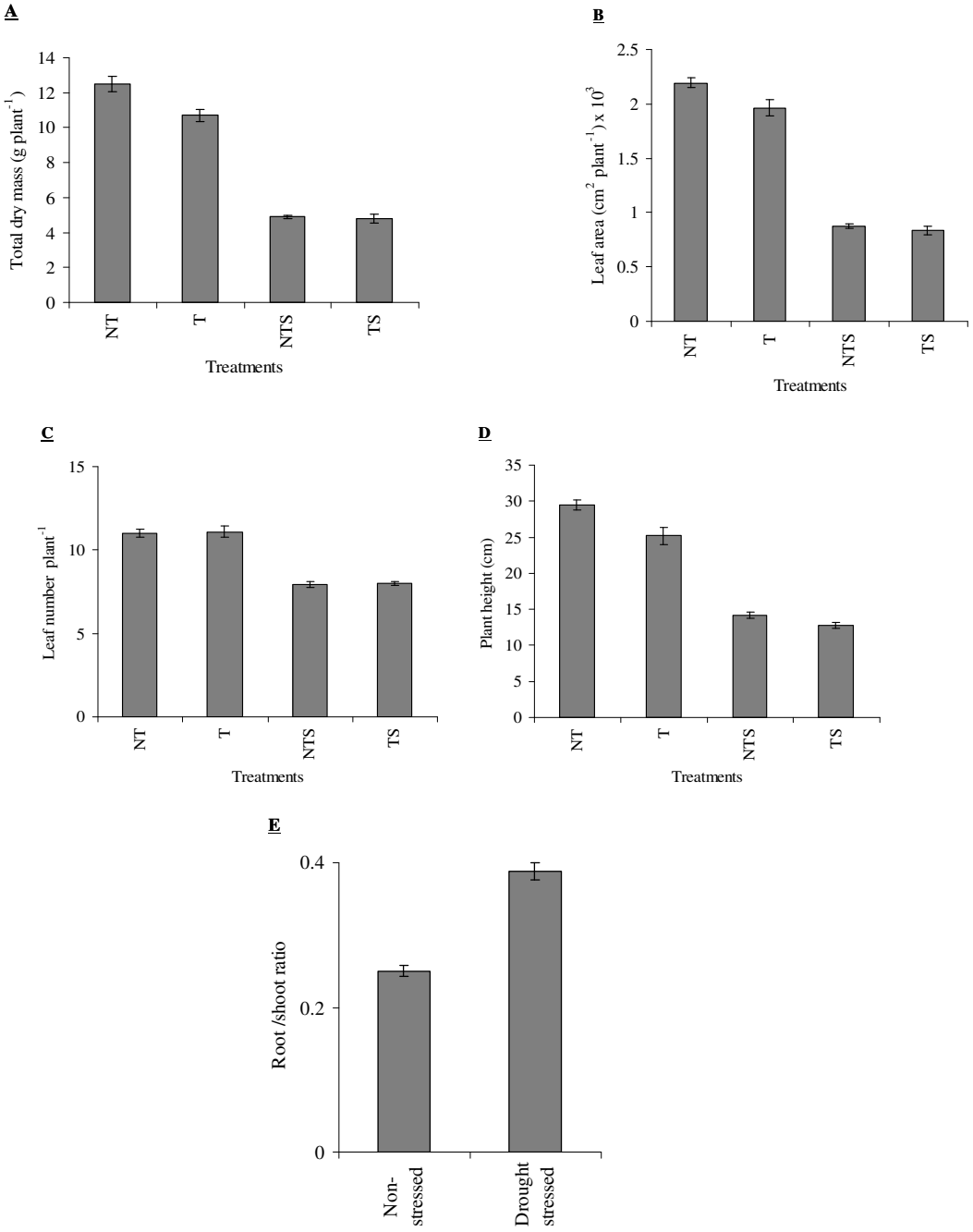


Figure 3.1 Growth characteristics of non-stressed, transformed (T) and non-stressed, non-transformed (NT) tobacco plants and drought-stressed transformed (TS) and drought-stressed non-transformed (NTS) tobacco plants. (A) Total plant dry mass, (B) leaf area, (C) leaf number, (D) plant height and (E) root/ shoot ratios of the different types of plants. Bars represent the mean of 32 plants ± standard error (S.E.).

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)

Treatments	Photosynthetic rate (P_n, µmol m^{-2} s^{-1})	Stomatal conductance g_s, mmol m^{-2} s^{-1}	Transpiration rate (E, mmol m^{-2} s^{-1})
Non-stressed			
Non-transformed	18.0±0.90	1.19±0.22	11.5±1.07
Transformed	16.1±0.82	0.81±0.26	9.5±0.77
Drought-stressed			
Non-transformed	4.1±0.55	0.027±0.005	0.94±0.17
Transformed	4.2±0.29	0.016±0.004	0.62±0.16

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

Growth performance of transformed and non-transformed tobacco plants was evaluated either under drought or heat stress or under a combination of both stresses. Drought and heat stress significantly ($P<0.05$) decreased total dry mass, leaf area, plant height, leaf number and leaf net photosynthetic activity. Plant total dry mass was reduced following drought stress by 68%, following heat stress by 44% and following a combination of both stresses by 79% (Figure 2A). Similarly, the leaf area was reduced by 67% (drought stress), 38% (heat stress) and by 82% (combination of both stresses) when compared to non-stressed plants (Figure 2B). Also, plant height was reduced following drought stress 48%, following heat stress by 48% and following a combination of both stresses by 75% (Figure 2C). Further, leaf number per plant was reduced by 12%, 50% and 60% by heat, drought and combination of both stresses, respectively, and net photosynthetic rate was reduced by 35% (heat), 64% (drought) and by 75% (combination of both heat and

drought stress) when compared to non-stressed plants. However, both growth characteristics and photosynthetic activity were not significantly different ($P>0.05$) between transformed and non-transformed tobacco plants when either drought, heat or a combination of both stresses was applied.

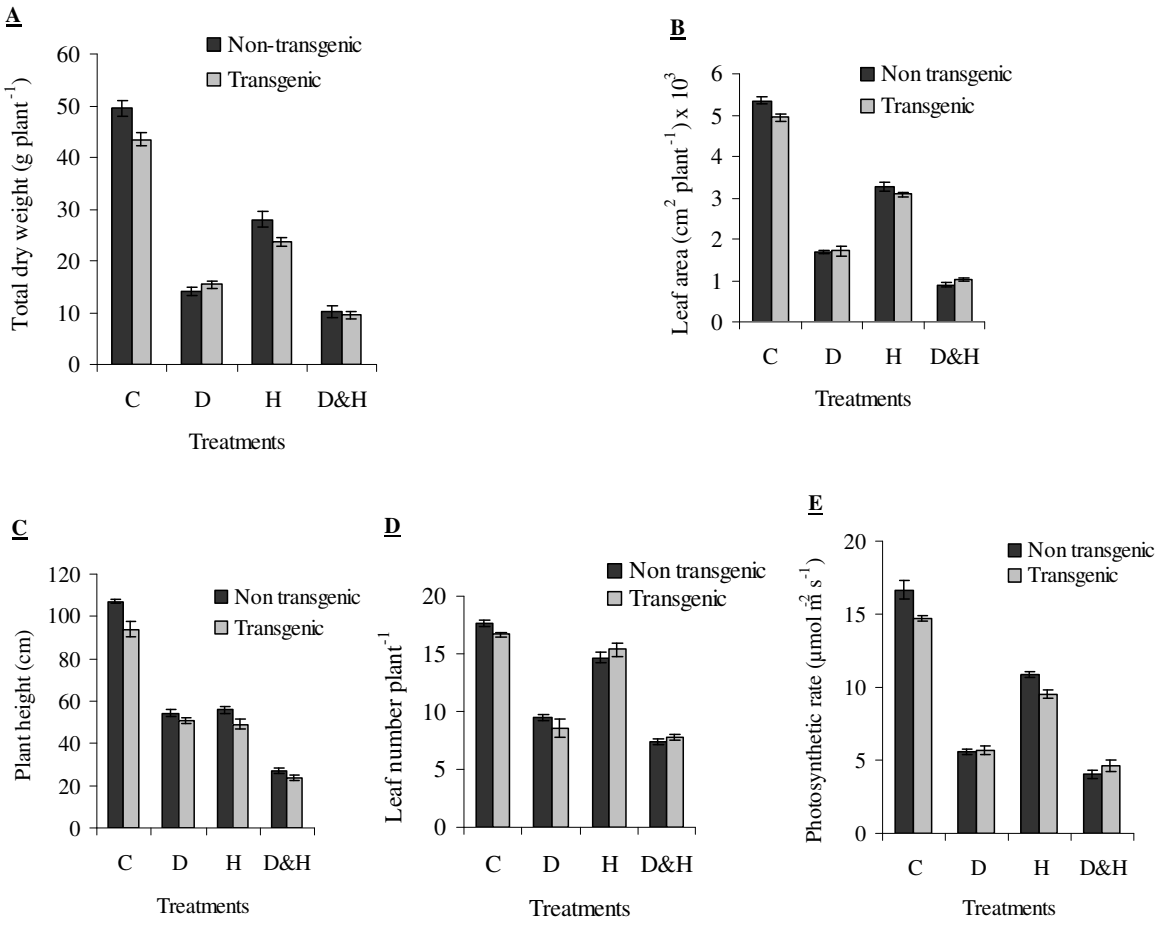


Figure 3.2 Plant growth and net photosynthetic rates of non-transformed and transformed plants under drought (D), heat (H) or a combination of both stresses (D&H). (A) Plant total dry mass, (B) leaf area (C), stem height (D), leaf number and (E) photosynthetic rate of transformed and non-transformed tobacco plants under non-stress (control), drought, heat and a combination of drought and heat stress. Bars represent mean of 8 plants ± standard error (SE).

3.5 Discussion

In this part of the study it was found that plant total dry mass, leaf number and area, stem height and photosynthetic rate were significantly reduced by drought, heat or a combination of these stresses. The effects of high temperature and drought stress on plant growth and productivity have been well documented in plants including tobacco, which has been also used in this study (Craufurd and Peacock, 1993; Savin and Nicolas, 1996; Jiang and Huang, 2001; Rizhsky *et al*., 2002). Biochemical and physiological alterations that occur during a combination of heat and drought stress on plants include enhancement of respiration and leaf temperature, reduction in photosynthesis, reduction/changes in type of antioxidant enzymes, which would lead to an increase in membrane lipid peroxidation (Jagtap *et al*., 1998; Jiang and Huang, 2001; Rizhsky *et al*., 2002). In tobacco, drought stress resulted in the suppression of both respiration and photosynthesis, whereas heat treatment resulted in enhanced respiration and stomatal conductance to lower leaf temperature by transpiration, but did not significantly alter photosynthesis (Rizhsky *et al*., 2002). In contrast, drought or a combination of drought and heat suppressed stomatal conductance raising the temperature of leaves (Rizhsky *et al*., 2002).

The present study also showed that net photosynthesis was significantly reduced by drought and combined drought and heat stress but less affected by heat stress. This also confirms the results reported by Jagtap *et al*. (1998) about the photosynthetic rate in sorghum where drought stress significantly reduced photosynthetic rates of different sorghum varieties when compared to heat or light stress. Further, in the present study it was also found that reduction in plant total biomass, leaf area and leaf number was more

severe than under heat stress. Reduction in growth is one of the most known effects of drought stress. It is mainly caused by inhibition of leaf and stem elongation when water the potential decreases below threshold. This differs among species and genotypes or cultivars within a species (Pelleschi *et al*., 1997; Younis *et al*., 2000). Also, the various mechanism by which drought stress reduces CO_2 assimilation and activity of photosynthetic enzymes includes, stomatal closure, the differences in the activation state of enzymes, decrease in the total protein content per leaf area or regulation at transcription, and translation of specific protein synthesis (Maroco, *et al*., 1999; Lawlor, 2002; Chaves *et al.*, 2002). Further, root growth is less sensitive to drought stress than stem growth (Creelman *et al*., 1990). This leads to an increase in root/shoot ratio that is commonly observed in plants exposed to drought stress and could also be confirmed in the present study. A decrease in available soil water decreases water uptake per unit root mass and may also reduce nutrient uptake, as delivery of nutrients by mass flow is hampered in dry soil (Poorter and Nagal, 2000; Marschner, 1995) resulting in overall growth reduction under drought stress.

However, in the present study it was found that, whatever growth parameter was measured following stress treatment, there was no significant difference in the response to stress between transformed and non-transformed plants. This was also true when the photosynthetic rate was measured after stress treatment. Under non-stress conditions in the greenhouse and growth cabinet, non-transformed plants even showed slightly higher total dry mass yield, stem height, leaf area and net photosynthetic rates than transformed plants. Therefore, expression of exogenous OC-I in transformed plants has no beneficial effect under drought or heat stress conditions when total dry mass, leaf area, leaf gas

exchange, leaf number and stem height were measured. Such an absence of difference in response between transformed and non-transformed tobacco plants might indicate an absence of interaction between exogenous OC-I and endogenous plant cysteine proteinases expressed following drought or heat stress. Such an absence of interaction could also emanate from differences in the localization of plant proteinases, the majority of which residing in the vacuole, and OC-I presumably expressed in the cytosol.

Since no highly significant differences in plant performance under drought or heat stress was found between the two types of plants, the influence of OCI expression on gene expression has been investigated to find out if both types of plants differ at all in gene expression. For that, cDNAs from heat-stressed transformed OC-I expressing and non-transformed plants were subtracted and identified cDNAs differently expressed between the two types of plant were used to study their transcription under heat stress.

CHAPTER FOUR

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

4.1 Abstract

Enhanced expression of proteinase inhibitor in transformed plant might confer multiple stress tolerance. The objective of this part of the study was to investigate differentially expressed gene(s) between oryzacystatin (OC-I) expressing transformed tobacco (Nicotiana tabacum L. cv. Samsun) and non-transformed tobacco plants by using cDNA Representational Difference Analysis (cDNA RDA). Three putative differentially expressed sequences were isolated from two weeks heat-treated transformed and non-transformed plants. Sequences included a fragment coding for a putative light harvesting chlorophyll a/b binding protein of photosystem II (LHC II) isolated from tester non-transformed plant DNA and a 60S ribosomal L12 like protein isolated from tester transformed plant DNA. A temporal expression study of the putative light harvesting chlorophyll a/b gene under heat treatment showed a difference in expression between transformed and non-transformed plants under non-stress conditions where the gene was down-regulated in transformed plants. Measurement of chlorophyll content and soluble proteins using two-dimensional gel electrophoresis also showed differences between the two plant types both under non-stress and heat stress conditions. This suggests that constitutive overexpression of OC-I transgene affects gene expression as a plant response to heat stress in tobacco.

4.2 Introduction

Representational Difference Analysis (RDA) was first described by Lisitsyn *et al*. (1993) for the identification of difference between two complex genomes. RDA belongs to the general class of DNA subtractive methodologies, in which one DNA population, known as the "driver", is hybridized in excess against a second population, which is the "tester", to remove common (hybridizing) sequences. Thereby "target" sequences are enriched that are unique to the "tester" population. cDNA RDA is a modification of the original RDA technique, in which the starting material is derived from mRNA rather than genomic DNA. Accordingly, targets are only genes which are expressed at the time total RNA is isolated (Hubank and Schatz, 1994 and 1999). The method is flexible, sensitive, and relatively inexpensive to perform. The method has further the major advantage that sequences common to both groups of cells are eliminated. This greatly simplifies the interpretation of results and identification of the differentially expressed genes. In addition, the exponential degree of enrichment achieved by the use of PCR in cDNA RDA enables the detection of very rare transcripts. Examination of differential gene expression using cDNA RDA requires the sampling of a population (of cells) grown under the condition(s) of interest and a population grown under conditions which differ only by those of interest. mRNA is extracted from both populations and used as a template for cDNA synthesis. The cDNA RDA technique has been successfully applied to isolate differentially expressed genes in rejuvenated soybean cotyledons (Ling *et al*., 2003), iron deficiency up-regulated genes in the bacteria *Neisseria meningitides* (Bowler *et al*., 1999) and garlic up-regulated genes in human gastric cancer cells (Li and Lu, 2002).

The goal of this part of the study was to investigate, by using cDNA RDA, possible effects of expression of exogenous OC-I on gene expression in transformed tobacco. In particular cDNA RDA was applied to isolate and characterize differentially expressed genes between transformed OC-I expressing and non-transformed tobacco plants after exposure of plants to stress and then to characterize these isolated gene sequences under stress conditions. Applying the RDA technique, a sequence coding for a chlorophyll *a/b* binding protein (LHC II) and an unknown sequences were isolated from heat-stressed non-transformed plant DNA, while a 60S ribosomal L12-like protein gene was isolated from heat-stressed tester transformed plant DNA.

4.3 Materials and Methods

4.3.1 mRNA isolation and cDNA synthesis

Total RNA was isolated from leaf samples collected from plants after two weeks of heat treatment. The fourth leaf from four individual plants, when leaves were counted from the top of the plant, was harvested and harvested leaves were mixed for total RNA isolation. Total RNA was extracted in TriPure total RNA isolation kit and any contaminant genomic DNA was digested by RNase free DNase (Roche, Germany). Total RNA was quantified using a NanoDrop® spectrophotometer and the quality of RNA was determined by running isolated RNA on a denaturing agarose gel containing ethidium bromide. About 250 µg of total RNA was used for mRNA purification using the Oligotex® mRNA isolation kit (QIAGEN GmbH, Hilden, Germany). Poly(A) RNA was then primed with Oligo$(dT)_{15}$ and reverse transcribed with AMV reverse transcriptase according to the manufacturer's recommendation (Roche, Germany) which was followed by second strand cDNA synthesis. The synthesised cDNA was visualized by running 4 µl of the reaction product on a 1% agarose gel containing ethidium bromide.

4.3.2 cDNA RDA

Preparation of amplicons and subsequent hybridisation and amplifications were done following the basic RDA procedure outlined by Lisitsyn *et al.* (1993) for genomic DNA and the modified version for differentially expressed genes (Hubank and Schatz, 1994).

4.3.2.1 Amplicon production

In the first step, cDNA (1 µg) derived from two types of tobacco, where one type served as tester cDNA and the other type as driver cDNA, was digested in a 20 µl restriction enzyme buffer at 37°C for 120 minutes with 10 units of the restriction enzyme *Mbo*I (Amersham, UK). After digestion, digested cDNA was analyzed for effective digestion on an ethidium bromide containing 1% agarose gel in TAE (Tris-Acetate-EDTA) buffer.

A pair of single-stranded oligonucleotide adaptors of different length was used to alter the ends of digested cDNA fragments to enable cDNA amplification. The longest adaptor was used as the primer for cDNA amplification after ligation. For adaptor ligation, digested tester and driver cDNA (between 0.5 and 1 µg) were mixed in a total volume of 60 µl with 7.5 µl of a 12-mer and a 24-mer adaptor with a concentration 58 pmol μl^{-1} (R-12 and R-24). Adaptor DNA was diluted from a 62 pmol μl^{-1} adaptor stock solution and the adaptor ligation reaction was carried out in a ligase buffer consisting of 66 mM Tris-HCl (pH 7.6); 6.6 mM $MgCl_2$; 10 mM DDT and 66 µM ATP. To anneal the adaptors, the ligation mixture containing cDNA fragments and adaptors was incubated in Eppendorf reaction tubes at 55°C for 1 minute in a heating block. After heating, the block was immediately placed into a cold room (4°C) for approximately 2 hours until the temperature dropped in the ligation mixture between 15°C and 10°C. The reaction tubes were then incubated on ice for 3 minutes. After incubation, 1 µl (5U μl^{-1}) of T4 DNA ligase (Amersham Life Science, UK) was added to the mixture and the ligation mixture was then incubated overnight at 14°C to ligate the adaptors.

Table 4.1 Oligonucleotide adapters and primers used for cDNA RDA

Adapter/Primer Name	Sequence
R-12	5'-GATCTGCGGTGA-3'
R-24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'
J-12	5'-GATCTGTTCATG-3'
J-24	5'-ACCGACGTCGACTATCCATGAACA-3'
N-12	5'-GATCTTCCCTCG-3'
N-24	5'-AGGCAACTGTGCTATCCGAGGGAA-3'
P-12	5'-GATCCAGATGTA-3'
P-24	5'-ATACGTGCAGGCTGGTTACATCTG-3'

For preparation of tester and driver amplicons by PCR, ligated DNA was diluted up to 200 µl with 140 µl of dsH_2O. For cDNA amplification, a PCR tube containing a PCR amplification mixture (100 µl), which contained 40 ng of ligated cDNA; 372 pmol of the 24-mer adaptor (R-24 Table 1); 10 mM dNTPs (4 µl); 25 mM $MgCl_2$ (6 µl) and PCR buffer consisting of 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$ and 0.001% w/v gelatine, was placed into a pre-warmed (72°C) thermocycler for 3 minutes (Gene amp PCR System, Perkin Elmer, USA). During this incubation, the 12-mer (R-12) dissociates, freeing the 3'ends for subsequent fill-in. To fill in the ends complimentary to the 24-mer adapters (R-24) the PCR machine was paused and 5 units of Taq DNA polymerase (5U μl^{-1}) (Amersham, Life Science, UK) and left for another 5 minutes. cDNA amplification by PCR was followed using 20 cycles of (60 seconds at 95°C; 3 minutes at 72°C) with the last cycle for DNA extension for 10 minutes at 72°C. Approximate total amount of cDNA of amplified tester and driver amplicons was determined on a 1.5% agarose gel in TAE buffer with sheared herring sperm DNA as a standard and NanoDrop (ND-1000) spectrophotometer to determine the total amount of amplified cDNA produced. Amplified cDNA was phenol/chloroform purified and after

ethanol precipitation (Sambrook *et al*., 1989) amplicon cDNA was dissolved in TE (10 mM Tris-HCl, pH 8; 0.1 mM EDTA) buffer to obtain a cDNA concentration of about 0.5 $\mu g\ \mu l^{-1}$.

4.3.2.2 First round subtraction and amplification

To cleave adaptors from amplified cDNA, driver cDNA and tester cDNA (90 µg) were digested for 1 hour at 37°C with 20 units of *Mbo*I μg^{-1} cDNA. Yeast glycogen carrier (10 µg) was added to digested cDNA, which was then purified using QIAquick (Qiagen, Germany) PCR purification kit to clean digested R-adapters and eluted with 70 µl of TE buffer to give a concentration of 0.5 $\mu g\ \mu l^{-1}$. The tester amplicon cDNA (1 µg) from which adaptors were cleaved was then ligated to a second adaptor pair (J-12 and J-24; Table 1) following the procedure outlined above for adaptor ligation. Ligated tester DNA was diluted to 10 ng/µl in a total volume of 70 µl with TE buffer. For hybridization, diluted tester cDNA 10 µl (100 ng) was mixed with 20 µl driver amplicon cDNA (10 µg) with a ratio of driver to tester of 100 to 1. Then 10 M ammonium acetate (12 µl) solution and 96% ethanol (144 µl) were added to the two cDNAs and mixed by sucking and blowing using an Eppendorf pipette. The mixture was incubated at -70°C for 10 minutes which was followed by an incubation period of 1 minute at 37°C. DNA was then precipitated by centrifugation for 10 minutes at 13000 rpm. and the cDNA containing pellet was washed twice with 70% ethanol and air-dried. The DNA pellet was re-suspended in 4 µl EE buffer containing 30 mM EPPS (N- (2-hydroxyethyl piperazine) - N-(3-propene sulfonic acid) (pH 8) and 3 mM Na_2 EDTA $2H_2O$. The cDNA was overlaid with 20 µl of sterile mineral oil and the sample was incubated at 98°C for 5 minutes to

denature the cDNA. The mix was cooled to 67°C and immediately 1 µl of 5 M sodium chloride solution was directly injected into the DNA drop and the mixture was incubated at 67°C overnight.

The mineral oil was removed and the sample was diluted by adding 200 µl TE buffer to the mixture. For initial amplification, diluted hybridized cDNA (40 µl) was added to 360 µl standard PCR reaction mixtures as outlined above for cDNA amplification. The solution was divided into 4 separate PCR tubes and 1µl of Taq DNA polymerase was added to each tube. The solution was incubated at 72°C for 5 minutes after which 4 µl of a 24-mer primer (J-24; Table. 1) were added to the solution. Eleven cycles of PCR (60 seconds at 94°C and 3 minutes at 70°C) were performed using an extension at 72°C for 10 minutes after the last cycle. To evaluate the effectiveness of the hybridization step, 20 µl of the hybridization mixture was amplified for an additional 32 cycles of amplification and any amplification products were visualized on an ethidium bromide containing 1.5% agarose gel in TAE buffer. If the amplification products were visible, 20 µl of the hybridization were digested with 20 units of mung bean nuclease at 30°C for 30 minutes to remove single-stranded DNA. The reaction was stopped by the addition of TE buffer (80 µl). The digested product was amplified in a standard PCR reaction mixture containing 4 µl of the 24-mer primer (J-24; Table 1). Amplified DNA subtraction products were purified with phenol/chloroform and precipitated with ethanol and finally dissolved in 200 µl of dsH_2O.

4.3.2.3 Second round subtraction and amplification

For the second round cDNA subtraction and kinetic enrichment by PCR, the first round cDNA subtraction products (5 µg) were digested with 50 units of *Mbo*I in a total volume of 100 µl. The digested DNA was purified with phenol/chloroform after addition of glycogen carrier (10 µg), ethanol precipitated and re-suspended in dsH_2O to obtain a cDNA concentration of 100 ng μl^{-1}. cDNA (200 ng) was ligated to a third set of adaptors (N-12 and N-24; Table1) in a total volume of 30 µl as described above for first round subtraction and amplification. The ligated cDNA was diluted to 1 ng μl^{-1} with addition of 130 µl of dsH_2O. cDNA hybridization and kinetic enrichment by a PCR reaction was carried out with 5 ng of ligated cDNA (5 µl) and an appropriate amount of driver amplicon cDNA (20 µl) as described above, in tester and driver ration of 1: 2000.

4.3.2.4 Third and fourth round subtraction and amplification

For the third round cDNA subtraction and kinetic enrichment by PCR, 100 ng of the second round subtraction products were ligated to a set of adaptors (P-12 and P-24, Pastorian *et al*., 2000, Table 1). Tester DNA (500 pg) was mixed with 10 µg of driver amplicon cDNA (20 µl) in a ratio of 1 to 20000, hybridized and amplified as described above. For the fourth round subtraction adaptors of the third round subtraction product were changed to a new adaptor set (J-12 and J-24; Table 1). The concentration of the ligated cDNA was adjusted to 10 pg μl^{-1} using consecutive dilutions with TE buffer. Before cDNA hybridization and kinetic enrichment of cDNA by PCR, hybridization and PCR amplification was carried out with 50 pg J-adaptors ligated cDNA and 10 µg of

driver amplicon in 20 µl low TE buffer as described above in a tester and driver ration of 1:200000.

4.3.2.5 Cloning and sequence analysis of difference products

Final RDA subtraction products were treated with appropriate restriction enzyme to remove ligated adaptors, separated on a 1.5 % ethidium bromide containing agarose gel in TAE buffer and visualized on a UV transluminator. cDNA fragments were eluted from the agarose gel and purified using a QIAquick gel purification kit (Qiagen). Purified DNA fragments were cloned into pGEM-T Easy vector system II (Promega, USA). Blue/white bacterial colony selection was made and isolated plasmids containing inserts were sequenced. Sequencing of the inserts were performed by using the BigDye® Terminator Cycle Sequencing FS Ready Reaction Kit, v 3.1 on ABI PRISM® 3100 automatic DNA-Sequencer (Applied Biosystems, Foster City, CA, USA). The BLASTN and BLASTX programs (Altschul *et al*., 1997) were used for homology search. Amino acid sequence alignment was made using Clustal W multiple alignment software (Thompson *et al.*, 1994).

4.3.4 Gene expression under heat stress

Eight weeks old tobacco plants raised from transformed and non-transformed plants were used for exposure to a high temperature. The heat treatment was achieved by moving 4 plants per treatment, as indicated in Figure 4.1, from a growth chamber maintained at 26/18°C (control) to a growth chamber maintained at a temperature of 38/30°C day/night (heat stress). The light photoperiod was set at 12 hours in both growth cabinets. Light in

growth cabinets was provided by a combination of incandescent and fluorescent light generating a photosynthetic photon flux density of 240 ± 10 µmol m^{-2} s^{-1}. The relative humidity in the growth cabinets during the study period was 70 ± 4%. Plants grown under 26°C day temperature and 20°C night temperature were considered to have been exposed to a standard control growth temperature. Watering was done on a daily basis and plants received a Hoagland nutrient solution three times per week. To minimize water deficit in the pots maintained at 38°C, plants were watered twice a day if required. Harvesting of leaves for analysis was done simultaneously for all four treated plants that have been exposed to heat for a varying time period. Leaf harvest was carried during the day photoperiod. Fully expanded leaves, (fourth leaf counted from the shoot apex) were harvested for all treatments and the experiments were repeated once. Harvested leaves were flash frozen in liquid nitrogen and either used immediately or stored at -80^{o}C until required. Leaf samples from both experiments were used for chlorophyll determination and as a source for RNA and protein.

4.3.5 Chlorophyll determination

The chlorophyll content of leaves was measured from three different plants per treatment. For determination of chlorophyll *a* and *b* content, the absorption of a 80% acetone extract containing the chlorophyll was measured at 663 and 645 nm in a spectrophotometer (Pharmacia LKB, Ultrospec III, UK) and the chlorophyll content was determined using absorption coefficients according to MacKinney (1941).

4.3.6 Two-dimensional gel electrophoresis (2-DE)

Leaf protein extraction and preparation for 2-DGE was performed as described by Salekdeh *et al*. (2002) with minor changes. A leaf protein extract from a leaf (0.3 g of fresh weight) was crashed in liquid nitrogen and rapidly homogenized in 1 ml of extraction buffer (20 mM Tris-HCl pH 8.0 containing 5 mM EDTA and 1 mM PMSF). The homogenate was transferred to 1.5 ml Eppendorf tube and centrifuged at 4°C for 10 minutes at full speed in an Eppendorf bench-top centrifuge. The supernatant containing the soluble protein was transferred to a new tube and centrifuged again as outlined above. The clear supernatant was precipitated with an equal volume of 10 % (w/v) trichloro-acetic acid containing 0.07% β-mercaptoethanol and cold acetone (-20°C) for 2 hours at -20°C and centrifuged for 15 minutes in an Eppendorf centrifuge at full speed. The resulting pellet was treated twice with cold acetone containing 0.07% β-mercaptoethanol following incubation for 1 hour at -20°C and centrifugation at 4°C. The pellet was finally freeze-dried. The protein pellet was then dissolved in lysis buffer (9.5 urea, 2% (w/v) a detergent CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), 0.8% (w/v) Pharmalyte pH 3-10 (Amersham), 1% (w/v) DTT). Approximately 200 µg protein was loaded onto a single immobilized pH gradient (IPG) gel strip (70 mm, pH 3-10, Amersham Pharmacia Biotech, UK). Iso-electric focusing (IEF) was conducted using the Pharmacia Multiphore system and the DryStrip kit (Amersham). IPG strips were re-hydrated in buffer (8 M urea, 0.5% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) IPG buffers) overnight before carrying out electrophoresis. IEF was performed at 500 V for 1 hour, followed by 1000 V for 1 hour, and 3000 V for 16 hour. The IPG gel strips were placed for 15 minutes in an equilibration buffer (6 M urea, 15 mM DTT, 30% glycerol and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8). A second equilibration was performed

with 2.5% iodoacetamide replacing DTT. The second dimension was run in vertical slabs (MINI PROTEAN II, Bio-Rad, USA) of 12% SDS-polyacrylamide gels. Duplicate gels were produced using same procedures from heat stressed and non-stressed transformed and non-transformed plants and gels were stained with Coomassie R250 or silver nitrate for detection of protein bands and compared visually.

4.4 Results

4.4.1 DNA RDA

A reciprocal cDNA RDA was used to identify gene sequences differentially expressed between transformed and non-transformed tobacco plants under stress conditions from cDNA population synthesized from mRNA obtained after two weeks of heat treatment. Figure 4.1 shows the amplicons (A) and difference products (DP) after the fourth round of subtraction (B) of heat-treated transformed and non-transformed plants. Sequential hybridisation and PCR amplification following the cDNA RDA procedure for differentially expressed sequences, as outlined by Hubank and Schatz (1994), allowed enrichment of difference products visible as distinct bands on an agarose gel (Figure 4.1 B).

Difference products were cloned and 70 cloned products were sequenced. BLAST search showed that the majority of the clones contained tobacco ribosomal RNA (28S, 26S, 23S, 18S and 16S) genes. Three products were identified as nuclear-encoded, two having a matching sequence on the database, and the third product with no matching sequence on the database. The two products (GBDP4-5d-12 and GBDP5-5d-11) were identified when cDNA from heat-stressed non-transformed plants were used as tester and cDNA from heat-stressed transformed plants as driver. The third difference product (clone GBDP4-3d-7) was isolated when cDNA from heat-treated transformed plant was used as a tester using cDNA from non-transformed plants as a driver. These three sequences were deposited on the Expressed Sequence Tag (EST) database with GenBank accession numbers CF931536, CF931537 and CF931538, respectively, for clones GBDP4-3d-7,

GBDP4-5d-12 and GBDP5-5d-11. Of the two cDNAs difference products from tester (non-transformed plant), the deduced amino acid sequence of one of the sequences (GBDP4-5d-12) with GenBank accession number CF931537 had a 98 – 100% identity with tobacco and other *Solanaceae* chlorophyll *a/b* binding protein sequences of photosystem II (Figure 4.2A), while the second 211 bp difference product (GBDP5-5d-11) with GenBank accession number CF931538 had no matching plant sequence on any public database. The third difference product (GBDP4-3d-7) with GenBank accession number CF931536, isolated from heat-stressed tester transformed plant was 324 bp long. By using a BLAST search, a matching plant sequence (90-97%, identity) was found, which is the 60S ribosomal L12 protein sequence of different plant species (Figure 4.2 B). However, this sequence had a very low (12%) similarity with known tobacco 50S ribosomal L12 sequences (EMBL accession number X62339, Elhag *et al*., 1992). Thus, this product might represent a novel nuclear-encoded tobacco chloroplast ribosomal protein sequence.

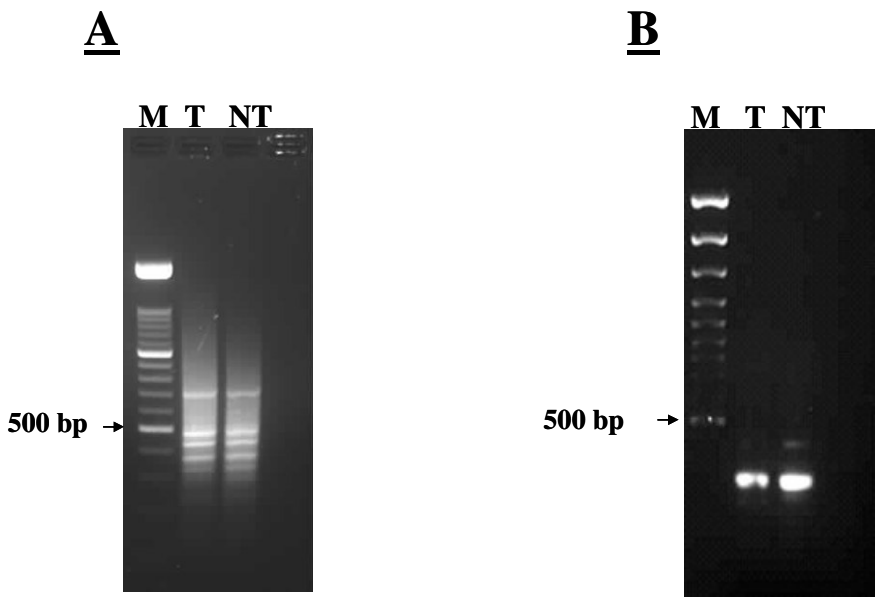
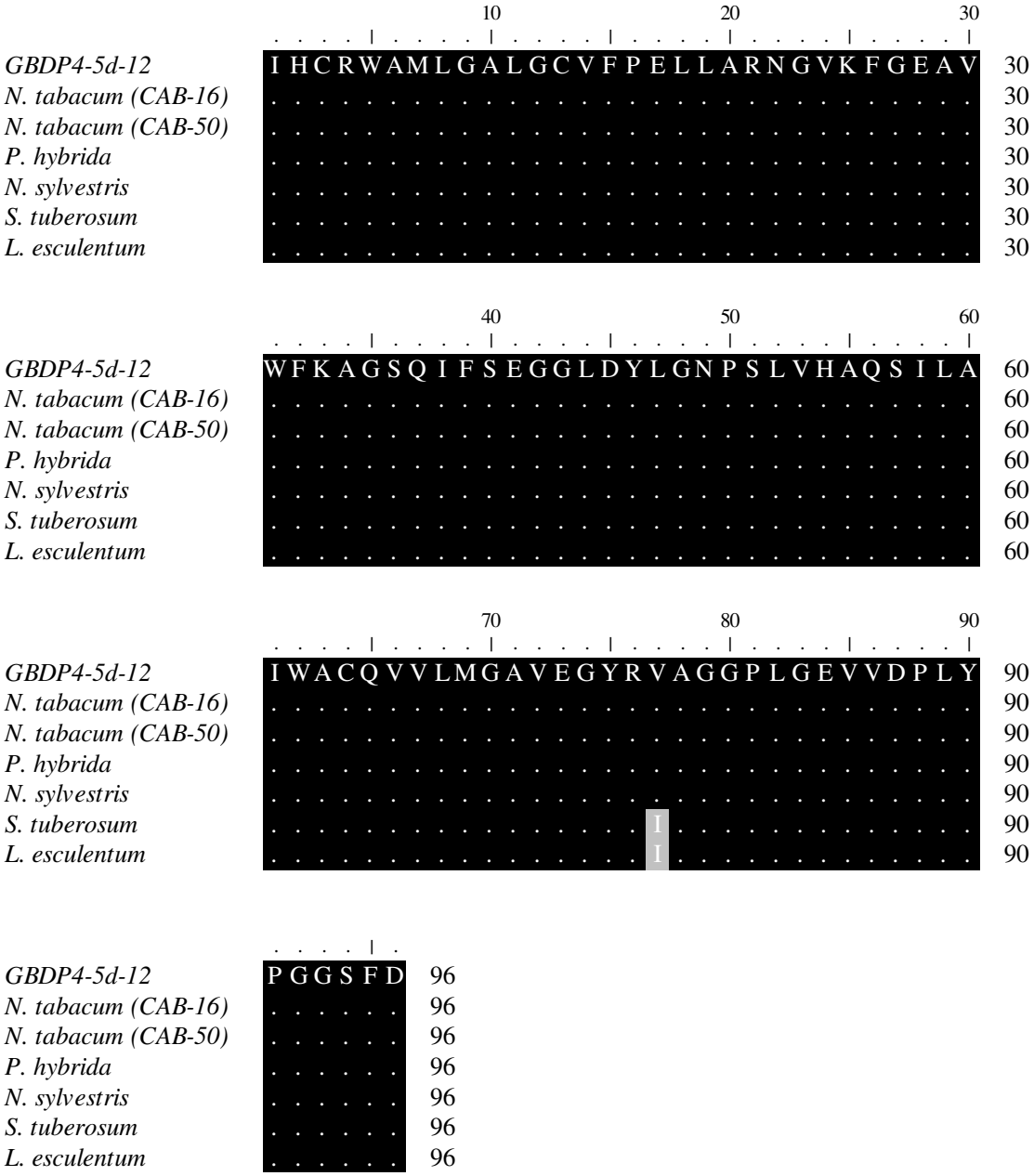


Figure 4.1 (A) Amplicons of heat-stressed transformed (T) and non-transformed (NT) plant for cDNA RDA. (B) Difference products after the fourth subtraction when cDNA of transformed plants were used as driver and non-transformed plant cDNA used as tester (lane T) and when cDNA of non-transformed plants were used as driver and cDNA of transformed plants was used as tester (lane NT). Ten µl of a PCR product was run on 1.5% agarose gel containing ethidium bromide for DNA visualization. M represents a 100 bp molecular weight marker purchased from Roche, Switzerland (A) and Fermentas, Litavia (B).

A



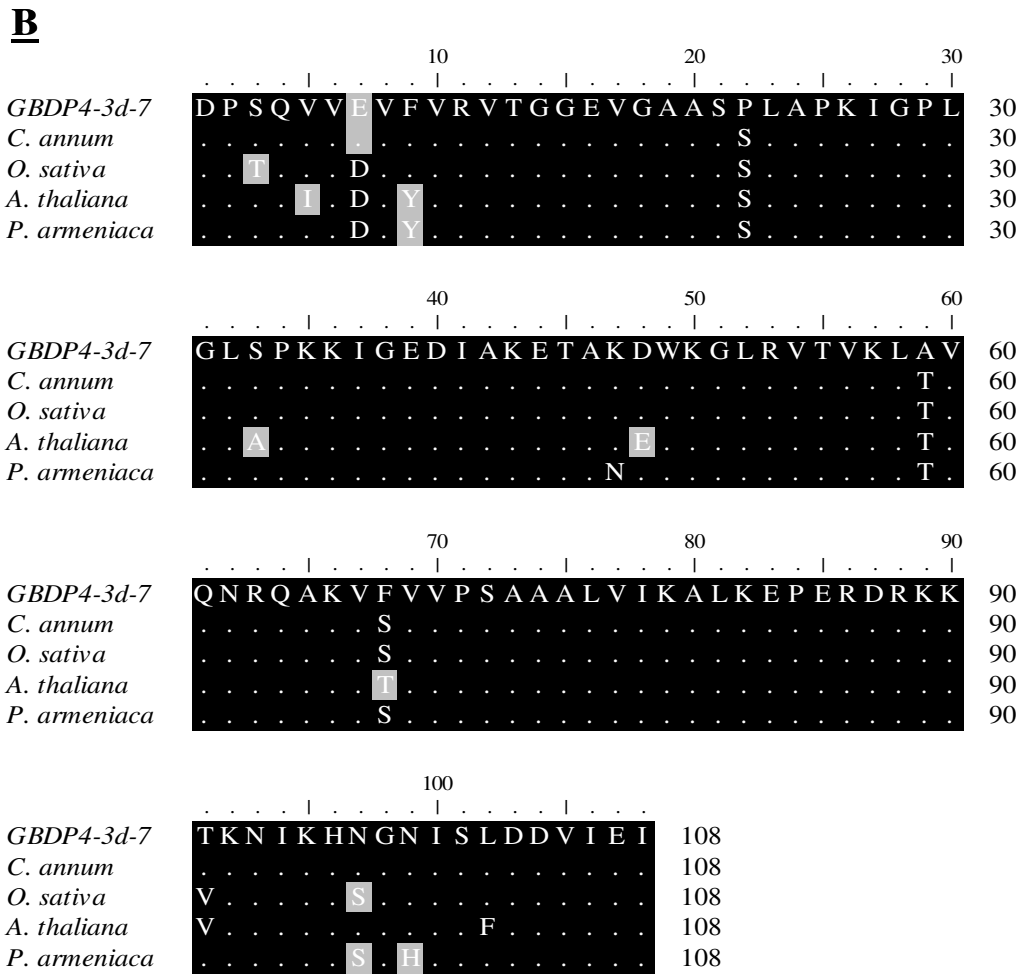


Figure 4.2 Alignment of deduced amino acid sequences. (A) GBDP4-5d-12 amino acid sequence alignment with the amino acid sequence of the plant light harvesting chlorophyll *a/b* binding protein derived from different plant species. (B) GBDP4-3d-7 amino acid sequence alignment with the L12 ribosomal protein amino acid sequences derived from different plant species. Identical amino acids are shown in dots, while similar amino acids (amino acids that have similar function) are light-shaded. Amino acid sequences in (A) represent *Nicotiana tabacum* cab 16 (P27492), *Nicotiana tabacum* cab 50 (P27496), *Petunia hybrida* cab 91R (P04783), *Nicotiana sylvestris* Lhcb1-7 (AB012639), *Solanum tuberosum* Lhcb1-2 (AAA80589) and *Lycopersicon esculentum* cab 1B (P07370). Sequences in (B) represent 60S ribosomal protein L12 from *Capsicum annum* (AAR83868), *Oryza sativa* (XP_467310), *Prunus armeniaca* (AAB97143) and *Arabidopsis thaliana* (AAM65708).

4.4.2 Expression of GBDP4-5d-12 Expression pattern of GBDP4-5d-12, which had a high homology to chlorophyll *a/b* binding protein of photosystem II, was further studied under non-stress and heat stress condition. A northern analysis was carried to determine the transcription of *GBDP4-5d*-12 (putative chlorophyll *a/b* binding protein gene, *lhcb1*) in transformed and non-transformed tobacco plants either under un-stressed conditions (C, control) or exposed to heat stress for a varying length of time. Transcription of the chlorophyll *a/b* binding protein gene (Figure 4.3) was lower in non-treated transformed plants expressing OC-I and also in OC-I expressing material until three days after heat treatment. Both types of plants showed, however, a decline in transcript level reaching a minimum 24 h after heat treatment. This was followed by an increase in accumulation at seven and fifteen days after heat treatment where transcript levels in both types of plants were almost identical. Increase in transcript levels might reflect an adaptive response to heat treatment not affected by expression of OC-I.

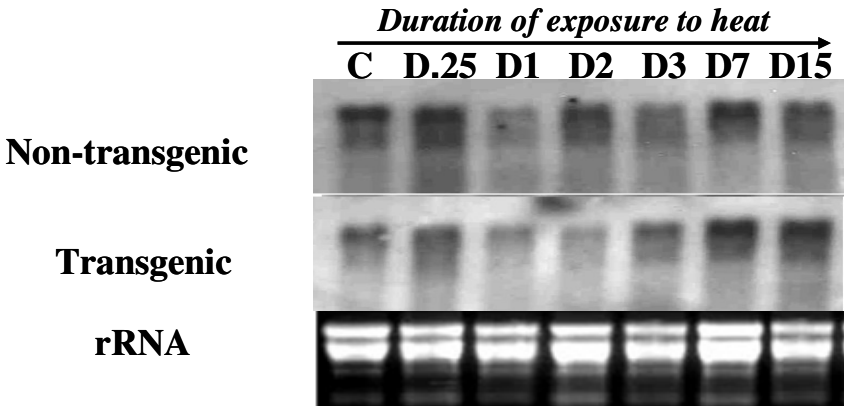


Figure 4.3 Temporal expression of a GBDP4-5d-12 under non-stress and during heat stress exposure. Total RNA was isolated from transformed and non-transformed tobacco plants that have been either non-stressed (C, control) or heat-stressed for various time periods. RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde transferred to Hybond™-N+ membrane and probed with *GBDP4-5d-12*. For heat exposure, plants were grown at 38°C/30°C (day/night) and control (non-stressed) plants were grown at 26°C/18°C (day/night) temperatures. Ethidium bromide stained rRNA was used to show equal loading of RNA and a degree of RNA degradation was evident in all samples.

4.4.3 Chlorophyll content

Leaf chlorophyll content was measured to demonstrate the extent of treatment effect. Measurements of leaf chlorophyll *a* and *b* contents were made under non-stress (control) and after 15 days of heat treatment. In both transformed and non-transformed plants,

chlorophyll *a* and *b* contents were significantly (P<0.05) higher under non-stress condition when compared to heat-treated plants (Figure 4.4 A and B). Non-transformed plants had significantly (P<0.05) higher chlorophyll *a* and *b* content than transformed plants under non-stress condition, but there was no difference in the chlorophyll content between the two plant types after 15 days of heat treatment.

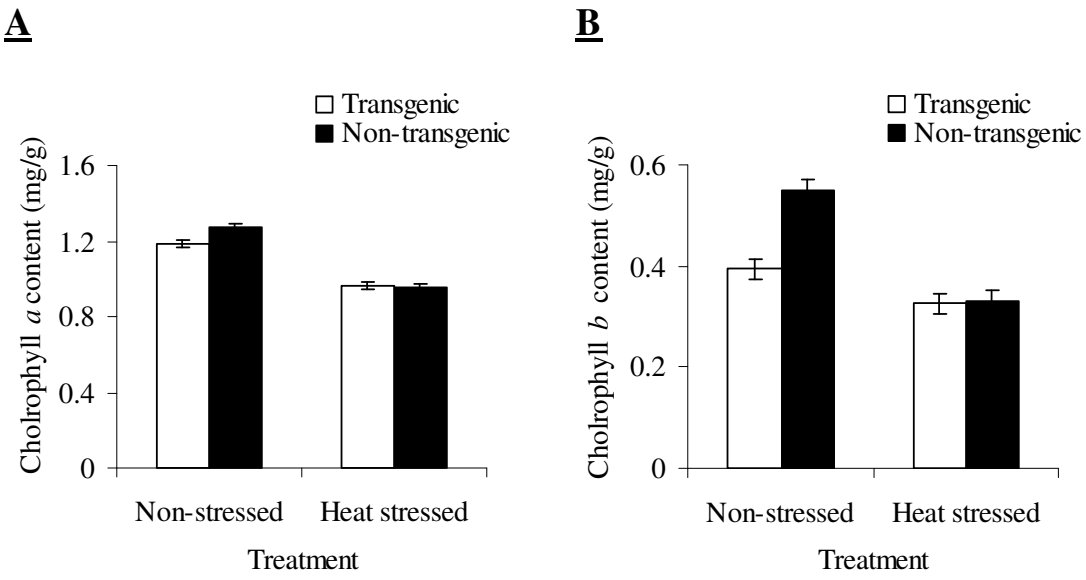


Figure 4.4 (A) Chlorophyll *a* and (B) chlorophyll *b* content (mg g^{-1} on fresh weight basis) of transformed and non-transformed tobacco leaves under non-stress and after heat stress exposure. Heat stress treatment was made at 38/30°C (day/night) temperature for 15 days, while non-stress treatment was at 26/18°C (day/night) temperature. Each data point represents the mean ± standard error (SE) of three independent observations.

4.4.4 Protein expression detected by 2-DE

To study the differential expression of leaf protein, protein extract from transformed and non-transformed plants were also compared by two-dimensional gel electrophoresis (2-DE) both under non-stress and after heat exposure for 15 days. Comparison of the spots on the gels from protein samples of transformed and non-transformed plants showed the presence of additional spots in transformed plant material which were absent or very weak in non-transformed plant material (boxed spots). Further, a single, visually distinguishable major band, appeared different between non-transformed and transformed plants (Figure 4.5 A and B; circled spots). The position of this single differentially expressed spot on the gel was compared with available data from the tobacco 2-DE database (http://tby2-www.uia.ac.be/tby2/). Comparison with other 2-DE from tobacco and other plant leaf proteins indicated that the abundant spot very likely contains the small subunit of Rubisco among possible other proteins. Proteins at this location were more abundant in non-transformed plants when compared to transformed plants under both non-stressed and heat-stressed conditions. The intensity of protein spots decreased under heat stress in both types of plants. However, the decrease is also somehow greater for non-transformed plants when compared to transformed plants (Figure 4.5 A and B). This possibly indicates a higher degree of protein degradation in non-OC-I expressing plants due to heat treatment.

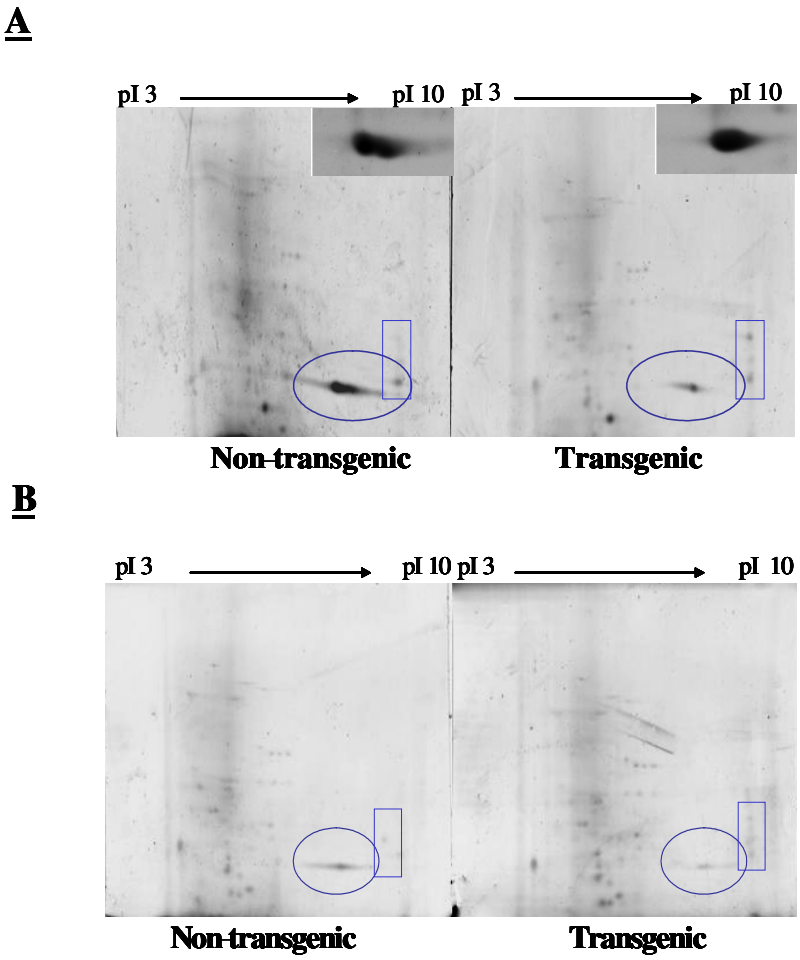


Figure 4.5 Two-dimensional gel electrophoresis of a plant leaf protein extract derived from an OC-I expressing (transformed) and a non-transformed tobacco plant. Coomassie blue-stained protein spots from (A) non heat-stressed and (B) heat-stressed non-transformed and transformed plants. Encircled spots represent major protein differences, and inserts in (A) are enlarged bigger spots showing possibly degradation in non-transformed plants.

4.5 Discussion

In general, cDNA RDA has been used for the isolation of differentially expressed genes between two populations of cDNAs. In principle the technique offers the advantage of isolating non-abundant, rare transcripts by using PCR amplification of differences. RDA has been further shown to be a reliable procedure for the discovery of target genes whose abundance differs in a magnitude of at least ten-fold. Nonetheless, the detection difference in abundance lower than that magnitude, although possible, is unpredictable (Hubank and Schatz, 2000).

cDNA RDA was applied in this study to isolate differentially expressed sequences from leaf cDNA preparations obtained from heat-stressed OC-I expressing and non-transformed tobacco plants. The technique was further applied for drought-stressed transformed and non-transformed plants and compared with non-stressed transformed and non-transformed plants. Cloning of the difference products after two rounds of hybridization and amplification in these experiments resulted in the isolation of 16S, 18S, 23S, 25S and 26S ribosomal RNAs. Primary attention to prove the usefulness of the technique in isolating unique difference products was then given to a single treatment, which was heat stress, and the rounds of hybridization and amplifications were increased to four following a standard procedure of cDNA RDA. At the same time the stringency in terms of driver/tester ratio was also increased. The increase in rounds of hybridization and amplification steps limited the appearance of rRNAs in the cloned difference products and resulted in the isolation of three sequences, which were two from non-transformed tester cDNA and one from transformed tester cDNA. Among them were a

nuclear-encoded chloroplast light-harvesting chlorophyll *a/b* binding protein gene of photosystem II that was isolated from non-transformed tester plants and a 60S ribosomal L12 like protein isolated from a tester transformed plant cDNA. Both of these sequences represent abundant transcripts encoded by multigene families in higher plants and are not rare transcripts. The third sequence isolated from tester non- transformed plant cDNA did not have a matching sequence on the database.

Of the three sequences expression pattern of one of the sequences (clone GBDP4-5d-12) that had higher identity with tobacco nuclear-encoded, the chloroplast light-harvesting chlorophyll *a/b* binding protein gene of photosystem II was studied in more detail. Light-harvesting chlorophyll *a/b* binding proteins are major components of the antenna complexes that collect and deliver light energy to the photosynthetic reaction centre in chloroplasts. The major complex (LHC II), which is associated with photosystem II, consists in higher plants of six types (Lhcb1–6) (Jansson, 1994). LHC II type I proteins (Lhcb1s) are encoded by the *lhcb1* multigene family, which is composed of eight members in *Nicotiana tabacum* L, with 95 – 99% protein identity and are expressed in the green part of the plant (Hasegawa *et al*., 2002). This fragment was initially isolated from two weeks heat-treated tester non-transformed plants in this study, and it was expected to have been more abundant in non-transformed plants after two weeks of heat treatment by northern analysis. By using northern blotting for detection, it was found in this study that the expression pattern of this gene differed between non-transformed and transformed plants until seven days after exposure to heat treatment and its transcript was more abundant in non-transformed plants than in transformed plants. This possibly might

indicate a protective effect of OC-I expressing plants on protein degradation processes whereby down-regulating transcription of this gene. However, it is rather unclear why this sequence was detected as a unique difference product using the RDA technique although the gene sequence was expressed in the two types of plants. There is the possibility that this abundant gene sequence was not completely removed during the hybridization process. The stringency at the first round of hybridization with a 100:1 driver: tester ratio has a typical 10% probability for appearance of false positive differences (Hubank and Schatz, 2000). By using then an amplification process all remaining un-hybridized DNA will be amplified resulting ultimately in the appearance of a false unique DNA subtraction product. In addition, the isolated *lhcb1* fragment also corresponds to the most conserved region within *lhcb1* gene family of tobacco and other plant species. Differential expression of the members of the family has been reported in tobacco under non-stress condition (Hasegawa *et al*., 2002). The possibility cannot be excluded that the gene sequence might have hybridized in the Northern blot procedure to all family members masking the actual differential expression of one or more members within the gene family.

In this study, under heat stress both chlorophyll content and intensity of protein spots decreased in the two types of plants. Reduction of chlorophyll content under heat stress has also been reported by Tewari and Tripathy (1998), which was caused by impaired biosynthesis of certain enzymes such as 5-aminolevulinic acid. The chlorophyll *a* and *b* contents of non-transformed plants were however higher than in transformed plants under non-stress conditions. This relates to the higher transcript level of the *lhcb1* found in non-

transformed plants. Both might have also contributed to the observed slightly higher photosynthetic activity and growth in non-transformed plants under non-stress conditions. In contrast, transformed plants had a lower transcript level for the chlorophyll *a/b* binding protein gene and also a lower chlorophyll content under non-stress condition. In addition, the intensity of certain protein spots on a 2D-gel was significantly lower in transformed plants when compared to non-transformed plants and also some proteins were protected additionally expressed under non-stress and stress conditions in OC-I expressing plants. Therefore, OC-I expression might have lowered protein turnover due to prevention of proteinase action resulting in a lower protein spot intensity and might also have protected a variety of expressed proteins against degradation.

The results obtained necessitated to design a strategy to identify tobacco proteinases that might be involved in the degradation of proteins and serve as a possible target for exogenous OC-I in a transformed plant. Therefore, in the following chapter cloning and characterization of two unique papain-like cysteine proteinases is presented as a first step to investigate possible OC-I cysteine proteinase targets in the tobacco.

CHAPTER FIVE

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

Beyene G, Kunert K, CH Foyer. 2006. Two new cysteine proteinases with specific expression patterns in mature and senescent tobacco (*Nicotiana tabacum* L.) leaves. *Journal of Experimental Botany* ***57:*** 1431-1443**.**

5.1 Abstract

Cysteine proteinases are involved in various physiological and developmental processes in plants. Two cDNAs from senescent and non-senescent tobacco leaves were isolated with degenerate primers designed from conserved regions of plant senescence-associated cysteine proteinases using rapid amplification of cDNA ends (RACE). Both sequences, the 1326 bp (*Nt*CP1) and the 1300 bp (*Nt*CP2) encode a full length papain-like cysteine proteinase. On the amino acid sequence level, *Nt*CP1 has a high similarity with other senescence-associated cysteine proteinases. It is expressed only in senescent leaves. It is not induced in mature green leaves upon exposure to drought or heat. These results suggest that it is might be a good developmental senescence marker in tobacco. In contrast, *Nt*CP2 has a high similarity to KDEL-tailed cysteine proteinases and is expressed in mature green leaves. Both drought and heat decreased *Nt*CP2 transcript abundance in mature green leaves. We conclude that *Nt*CP1 is a senescence specific cysteine proteinase whereas *Nt*CP2 fulfils roles in green leaves that might be similar to those of KDEL-tailed cysteine proteinases involved for example, in programmed cell death.

Key words: Cysteine proteinase, KDEL motif, senescence markers, tobacco

*Nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank under accession number AY881010 and AY881011.

5.2 Introduction

Cysteine proteinases are involved in a variety of proteolytic functions in higher plants (Granell *et al*., 1998). Many of the endogenous plant cysteine proteinases identified to date have acidic pH optima *in vitro*, suggesting that they are localized to the vacuole *in vivo* (Callis, 1995). Cysteine proteinase expression has been intensively studied with various expression patterns reported for different stages of plant development (Buchanan-Wollastan and Ainsworth, 1997; Guerrero *et al*., 1998; Xu and Chye, 1999). Such proteinases are involved in processing and degradation of seed storage proteins (Shimada *et al*., 1994; Toyooka *et al*., 2000), fruit ripening (Alonso and Granell, 1995) as well as in legume nodule development (Naito *et al*., 2000). They are also implicated in responses to stresses such as wounding, cold and drought (Schaffer and Fischer, 1988; Koizumi *et al*., 1993; Linthorst *et al*., 1993; Harrak *et al*., 2001) as well as in programmed cell death (Solomon *et al*., 1999; Xu and Chye, 1999). Some cysteine proteinases have specific characteristics such as a C-terminal KDEL motif. This motif, which is an endoplasmic reticulum retention signal for soluble proteins, allows cysteine proteinase propeptides to be stored either in a special organelle, called the ricinosome (Schmid *et al*., 1999), or in KDEL vesicles (KV) before transport to vacuoles through a Golgi complex-independent route (Okamoto *et al*., 2003).

A number of genes encoding papain-like cysteine proteinases have also been isolated from senescing organs including leaves (Lohman *et al*., 1994; Ueda *et al*., 2000; Gepstein *et al*., 2003), flowers (Eason *et al*., 2002), legume nodules (Kardailsky and Brewin, 1996) and germinating seeds (Ling *et al*., 2003). In general, senescence is characterized by the

breakdown of proteins (Callis, 1995) in senescing organs and nutrient remobilization to other developing parts of the plant (Noodén, 1988). There is considerable evidence from screening of cDNA libraries derived from senescent leaf tissues that the expression of the vast majority of genes is down regulated during senescence (Bhalerao *et al*., 2003). Senescence down-regulated genes (SDGs) include photosynthesis genes such as those encoding the chlorophyll a/b binding protein and the ribulose-1, 5-bisphosphate carboxylase-oxygenase (Rubisco) small subunit (Humbeck *et al*., 1996). However, a number of senescence-associated genes (SAGs) are up-regulated during leaf senescence (Lohman *et al*., 1994; Quirino *et al*., 1999; Swidzinski *et al*., 2002; Gepstein *et al*., 2003; Bhalerao *et al*., 2003; Lin and Wu, 2004). These SAGs genes are either expressed exclusively during senescence (Class I SAGs) or their expression increases during senescence from a continuous basal level during leaf development (Class II SAGs) (Gan and Amasino, 1997). Of the few SAGs that are highly senescence-specific, SAG12, encodes a cysteine proteinase. It is highly abundant in senescing leaves but is undetectable in non-senescent leaves (Lohman *et al*., 1994).

Although cysteine proteinases have been extensively characterized in Arabidopsis, only some cysteine proteinases have been investigated in other plant species. In tobacco, several cysteine proteinases have been identified (Linhorst *et al*., 1993; Ueda *et al.,* 2000; Senyuk *et al*., unpublished, Gene bank accession number: CAB44983). However, neither KDEL nor exclusively senescence-related cysteine proteinases have been so far isolated and characterized from tobacco.

The objective of this study was therefore to isolate development-related cysteine proteinase genes from tobacco. Here we describe the isolation and characterization of two novel tobacco cysteine proteinase coding sequences. These sequences, termed *Nt*CP1 and *Nt*CP2, were isolated from senescent and mature green tobacco leaves, respectively. *Nt*CP1 and *Nt*CP2 are differentially expressed in response abiotic stress. They belong to two distinct subgroups within the papain-like family of cysteine proteinases. In addition, they are phylogenetically distant to other tobacco cysteine proteinase coding sequences described to date.

5.3 Materials and methods

5.3.1 Plant material

Nicotiana tabacum L. cv. Samsun plants were grown in a greenhouse and maintained at a 26/20ºC day/night temperature cycle and a 12/12 hours light/dark cycle. Photosynthesis photon flux density during light phase was 600 ± 50 µmol $m^{-2}s^{-1}$ and plants were grown at a relative humidity of 60%. For measurements, senescent and non-senescent, fully expanded mature green leaves from three-month old plants were used. Non-senescent, green leaves used in these experiments had the highest chlorophyll content of all leaves on the plant and they had no visible symptoms of yellowing. Leaves were considered senescent when they showed yellowing and their chlorophyll content was lower than 40% of the chlorophyll content of a green mature leaf without signs of yellowing.

5.3.2 Plant treatment

For drought stress, watering of tobacco plants was withheld for ten days. For heat stress, plants were moved to a growth chamber maintained at temperature of 38/28 ± 2°C day/night temperature cycle for ten days. A combination of heat and drought stress was carried out by withholding water for three days from plants grown at 38/28 ± 2°C. For all stress experiments, photosynthesis photon flux density in the growth chambers was 300 ± 20 µmol $m^{-2}s^{-1}$. For analysis, leaf samples were collected from stressed and non-stressed plant of the same age and samples were either immediately used after freezing in liquid nitrogen or kept after freezing by storing at -80°C until needed.

5.3.3 Chlorophyll determinutesation

Leaf chlorophyll content of leaves was measured from three leaf discs each with 8 mm diameter. Spectrophotometric determination of total chlorophyll content in 80% acetone was done according to the calculation described by MacKinney (1941).

5.3.4 Proteinase determination

For determination of proteinase activity, leaf samples were homogenized in extraction buffer (50 mM Tris-HCl, pH 7.4) in the presence of liquid nitrogen without the addition of a proteinase inhibitor during homogenization. Samples stored on ice were processed immediately and all extraction steps were performed on ice to minimize any proteinase action. Homogenates were centrifuged at 13000 rpm at 4°C for 15 min and the supernatant was used for the different assays. Protein content of supernatant was quantified according to the method described by Bradford (1976) using BSA as a standard.

5.3.5 Proteinase determination with gelatine SDS-PAGE

Proteinase containing supernatants from leaf homogenates with 10 µg of total protein were added to an equal volume of a loading buffer (90 mM Tris-HCl, pH 6.8; 20% glycerol; 2% SDS and 0.2% bromophenol blue). To determine the proteinase composition in the samples, different proteinase inhibitors were added to protein samples before the addition of loading buffer. Samples were incubated with the different inhibitors for 15 minutes at 37°C with final concentrations of 100 µM of E-64 (inhibitor of cysteine proteinases), 100 µM of BBTI (soybean Bowman-Birk inhibitor of serine proteinases) or 5 mM of PMSF (inhibitor of serine and cysteine proteinases). After

incubation, samples were separated on a 10% resolving sodiumdodicylsulphate polyacrylamide (SDS-PAGE) gel (Laemmli, 1970) containing 0.1% gelatine (type I from porcine skin) (Sigma, USA) with a 5% staking gel according to the method outlined by Michaud *et al.* (1993b). Gels were run at 150 V for one hour at 4°C. Proteinases present on the gels were re-natured in a 2.5% Triton X-100 solution at room temperature with gentle shaking for 30 minutes. Respective proteinase inhibitors at similar concentrations as those used for sample incubations were added to the proteinase re-naturation and development buffers. However, all gels containing inhibitor-free samples were treated with proteinase inhibitor free buffers for loading, re-naturation and development. After proteinase re-naturation, gels were rinsed in an excess of distilled water and developed overnight at 37°C in proteolysis buffer (100 mM citrate phosphate buffer, pH 6.0; 10 mM L-cysteine). Gels were then stained in 0.05% Coomassie Briliant Blue R-250 in 10% acetic acid and 25% iso-propanol and de-stained with the same solution without addition of the dye. Areas of proteinase activity were identified as clear bands against a blue background.

5.3.6 Azocasein assay

Total proteolytic activity of leaf extracts from senescent leaves was determined using azocasein (Sigma, USA) as a proteinase substrate according to the method of (Hines *et al.*, 1992). Different extract samples (50 µl/sample) containing 50 µg protein were incubated in 200 µl proteolysis buffer (100 mM citrate phosphate buffer, pH 6.0 and 10 mM L-cysteine) for 30 minutes at 37°C with and without the addition of proteinase inhibitors. Inhibitors with final concentrations in the incubation mixture were E-64 and

BBTI and pepstatin A each at 100 µM, PMSF at 5 mM, EDTA at 20 mM. Azocasein (2%) was dissolved in proteolysis buffer and 200 µl of azocasein solution was then added to the incubation mixture and incubated for 24 hours at 37°C. After incubation, the reaction was stopped by adding an equal volume of 10% trichloroaceticacid (TCA) to the reaction mixture, which was followed by incubation on ice for 30 minutes and centrifugation of mixture at 12000 g for 5 minutes. After centrifugation, the supernatant (500 µl) was added to an equal volume of 1M NaOH for colour development and the absorbance of the mixture was measured at 440 nm in a spectrophotometer. As blanks, identical reactions were set up but reactions were immediately stopped by addition of TCA.

5.3.7 Fluorimetric measurement of cysteine proteinase activity

Cysteine proteinase activity was measured with the fluorescence substrate Z-phe-arg-AMC with or without addition of a cysteine proteinase inhibitor using a modified method described by Abrahamson (1994). For determination, a plant protein extract (50 µl) with 30 µg of protein/sample was mixed with 325 µl proteolysis buffer (100 mM citrate phosphate pH 6.0 and 10 mM L-cysteine). The mixture was pre-incubated for 10 minutes at 37°C with or without addition of a cysteine proteinase inhibitor (E-64 at 100 µM and PMSF at 5 mM) before adding 125 µl of proteinase substrate (20 µM Z-phe-arg-AMC) diluted in proteolysis buffer. Similar reactions were set without plant extract as a blank. The reaction mixture was then incubated for 10 minutes at 37°C and was stopped by the addition of 1.0 ml stopping buffer (10 mM sodium monochloroacetate; 30 mM sodium acetate; 70 mM acetic acid, pH 4.3). Release of fluorescent AMC was determined using a

fluorescence spectrophotometer (Model F-2000; Hitachi, Japan) using an excitation and emission wavelength of 370 nm and 460 nm, respectively.

5.3.8 Isolation of cysteine proteinase coding sequences

Total RNA was extracted from leaf material using the TriPure total RNA isolation kit according to the manufacturer's recommendation (Roche, Germany) and contaminant genomic DNA was digested by RNase-free DNase. Total RNA (10 µg) was used for cDNA synthesis using oligo-$(dT)_{15}$ for priming poly(A) RNA and AMV reverse transcriptase for reverse transcription followed by second strand synthesis according to the outline given by the manufacturer (Roche, Germany). Synthesized double-stranded cDNA was used as a template for gene isolation by the polymerase chain reaction (PCR).

For isolation of cysteine proteinase coding sequences from synthesized cDNAs, a forward degenerate primer with the sequence 5'-AGAATCAAGGACAATGTGGATGY(C/T)TGY(C/T)TGGGC-3' and a reverse degenerate primer with the sequence 5'-TCCCCAAGAATTCTTAATAATCCAR(A/G)TAY(C/T)TT-3' were used. Design of both primers was based on sequence information for the conserved regions of senescence-associated cysteine proteinases from *Brassica napus* (GenBank accession number AAD53011), *Arabidopsis thaliana* (GenBank accession number AAK64131) and *Gossypium hirsutum* (GenBank accession number AAT34987) using the CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primers) program (Rose *et al.*, 2003). For amplification of coding sequences by PCR, a primer annealing temperature of 50°C was used in a standard PCR reaction containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl,

0.25 mM each dNTPs, 5 units of Taq polymerase (Roche, Germany) and 0.2 µM of the degenerate primer mixture. The PCR cycles were 2 minutes at 94°C and followed by 35 cycles at 30 seconds at 94°C, 50°C 30 seconds and 72°C 60 sec followed by an extension at 72°C for 10 minutes.

Full-length cDNA clones for cysteine proteinases were obtained by performing 5' and 3' RACE using the GeneRacer™ kit according to the manufacturer's instruction (Invitrogen, USA) along with gene-specific primers. Gene-specific forward primer 5'-CATGGCTGAAGGTGGCGAGTGTGA–3' and two nested reverse primers with the sequences 5'-CCTTAGGTGCTGTTGCAGGAGACCCTGT-3' (external primer) and 5'-CATTCAGGTCCCCACGAGTTCCTCAC–3' (internal primer) were used for isolation of a full-length cysteine proteinase coding sequence from synthesized cDNA derived from non-senescent leaf material. Two forward primers with the sequences 5'-TTCATGGGGCAGTAAATGGGGTGACA-3' (external primer) and 5'-TGGGGCAGTAAATGGGGGACAGTGG-3' (internal primer) and a reverse primer of 5'- GCCTTCTCACTG TTCGCTGGCACA -3' were used for isolation of a full-length cysteine proteinase coding sequence from synthesized cDNA derived from senescent leaf material. The 5' RACE, 5' nested, 3' RACE and 3' nested primers were provided with the GeneRacer™ kit (Invitrogen, USA) that were used together with the gene specific primers. All amplified PCR products were finally cloned into the vector pGEM-T Easy vector system II (Promega, USA).

5.3.9 Sequence analysis

Sequencing of the inserts were performed by using the BigDye® Terminator Cycle Sequencing FS Ready Reaction Kit, v 3.1 on ABI PRISM® 3100 automatic DNA-Sequencer (Applied Biosystems, USA). The BLASTN and BLASTP programs (Altschul *et al*., 1997) were used for gene sequence homology search. Amino acid sequences of selected plant papain-like cysteine proteinases including known *N. tabacum* proteinases were aligned using Clustal W (Thompson *et al*., 1994). Phylogenetic tree was constructed from aligned sequences using maximum likelihood parsimony with 100 bootstrap re-sampling methods of the Phylip 3.6 package (Felsenstein, 1989). ExPASy (Gasteiger *et al*., 2003) web site and programs therein were used for prediction of amino acid features of *Nt*CP1 and *Nt*CP2.

5.3.10 Southern blot analysis

Genomic DNA (20 µg) was digested for 12 hours with 100 units of *Eco*RI and *Xba*I and digested DNA was separated on a 1% (w/v) agarose gel at 50 V for 5 hours. Separated DNA was transferred to a Hybond N+ membrane using a standard protocol as outlined by Sambrook and Ressell (2001). Gene specific DNA probe for *Nt*CP1 was prepared by amplifying a 659 bp fragment and probe for *Nt*CP2 was prepared by amplifying a 604 bp DNA fragment. Probes were labelled using a random-prime labelling kit according to the manufacturer's instruction (Amersham, UK). Pre-hybridization for 2 hours and hybridisation of probes with membrane-bound DNA were performed overnight at 60°C in a hybridisation buffer containing 0.5 M Na_2HPO_4, pH 7.2, 7 % (w/v) SDS and 1mM EDTA. Three subsequent stringency washes were performed at 60°C for 15 minutes

each. The first washing solution contained 0.1% SDS (w/v), 2 x SSC, the second 0.1% SDS (w/v), 1 x SSC and the third washing 0.5 x SSC and 0.1% SDS. Detection of hybridization products was carried out with the Gene Images™ CDP- Star™ system (Amersham, UK) followed by exposure to a Hyper™ film (Amersham, UK).

5.3.11 Northern blot analysis

Total RNA was extracted from leaf material using the TriPure total RNA isolation kit according to the manufacturer's recommendation (Roche, Germany) and contaminant genomic DNA was digested by RNase-free DNase. Northern blotting was carried out as described in Sambrook and Russell (2001). For blotting, total RNA (20 µg) was first size-separated on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to Hybond-N+ membrane (Amersham, UK) and then UV cross-linked. Pre-hybridisation and hybridisation of RNA containing membranes was carried out at 65°C. Specific probes for *Nt*CP1 and *Nt*CP2 were produced by PCR amplification of a *Nt*CP 1 fragment (positions 500 – 833; see Figure 5.3A) and a 596 bp *Nt*CP2 fragment (positions 705 –1300; see Figure 5.3B) from cloned products and labelling of probes using a random-prime labelling kit according to the manufacturer's instruction (Amersham, UK). Hybridization and stringency washes were carried out as outlined under Southern blot analysis. As an internal control for equal loading, a 598 bp *N. tabacum* 18S ribosomal RNA probe (GenBank accession number AJ236016) was amplified from genomic DNA of tobacco using the forward primer 5'–CCTGAGAAACGGCTACCACATCCA-3' and reverse primer 5'–CGAGCCCCCAACTTTCGTTCT-3'.

5.3.12 Statistical analysis

All estimates of sample variability are given in terms of the SD of the mean. The significance of differences in chlorophyll and soluble protein content and proteinase activity of tobacco leaves with different age was determined by the Student's two-tailed *t* test. P values ≤ 0.05 were considered significant.

5.4 Results

5.4.1 Leaf chlorophyll, soluble protein content and proteinase activity

Tobacco leaves were harvested from plants at the development stage shown in Figure 5.1A. The leaves were ranked from the top of the plant to the bottom. The third and the seventh leaf were denoted as markers for green and senescent leaves, respectively (Figure 5.1B). Leaf number 7 had significantly less ($p \leq 0.05$) chlorophyll (60%) and protein (49%) than leaf number 3 (Figure 5.1C).

Senescent leaves (S) had higher maximal extractable proteinase activities than mature green (G) leaves (Figure 5.2A) when assayed by the gelatine SDS-PAGE method. Addition of either the cysteine proteinase inhibitor, E-64 or a serine proteinase inhibitor (BBTI) inhibited proteinase activity in extracts from both types of leaves. Treatment of plant extracts with PMSF, which inhibits cysteine and serine proteinases, completely inhibited proteinase activity in both types of leaf extract (data not shown).

Similarly, the addition of E-64, BBTI, the aspartic-proteinase inhibitor, pepstatin A or PMSF to leaf extracts, analysed in the azocasein assay system significantly inhibited activity ($p \leq 0.05$) by 35%, 51%, 13% and 72%, respectively (Figure 5.2B). No inhibition of activity was observed when EDTA was added to extracts to prevent metallo-proteinase activity. This indicates that senescent tobacco leaves have at least three different classes of proteinase activity (cysteine, serine and aspartic proteinases) but metallo-proteinases were not detected under this experimental system.

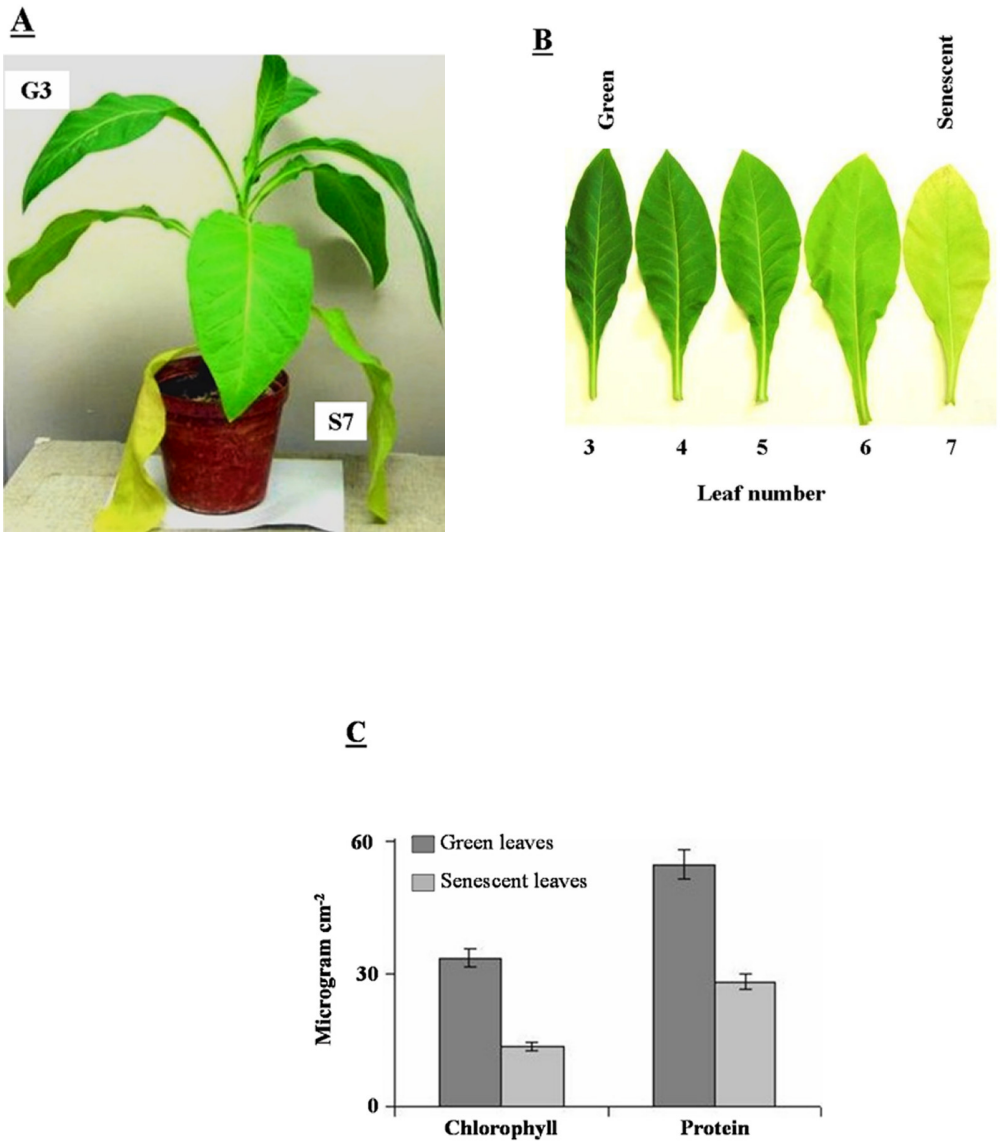


Figure 5.1 (A) Tobacco plant and (B) developmental stages of leaves with progression of natural senescence. For the study fully expanded mature green (G3) and senescent leaves (S7) were used. (C) Soluble protein and chlorophyll content of mature green (G3) and senescent (S7) leaf material. Data represent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

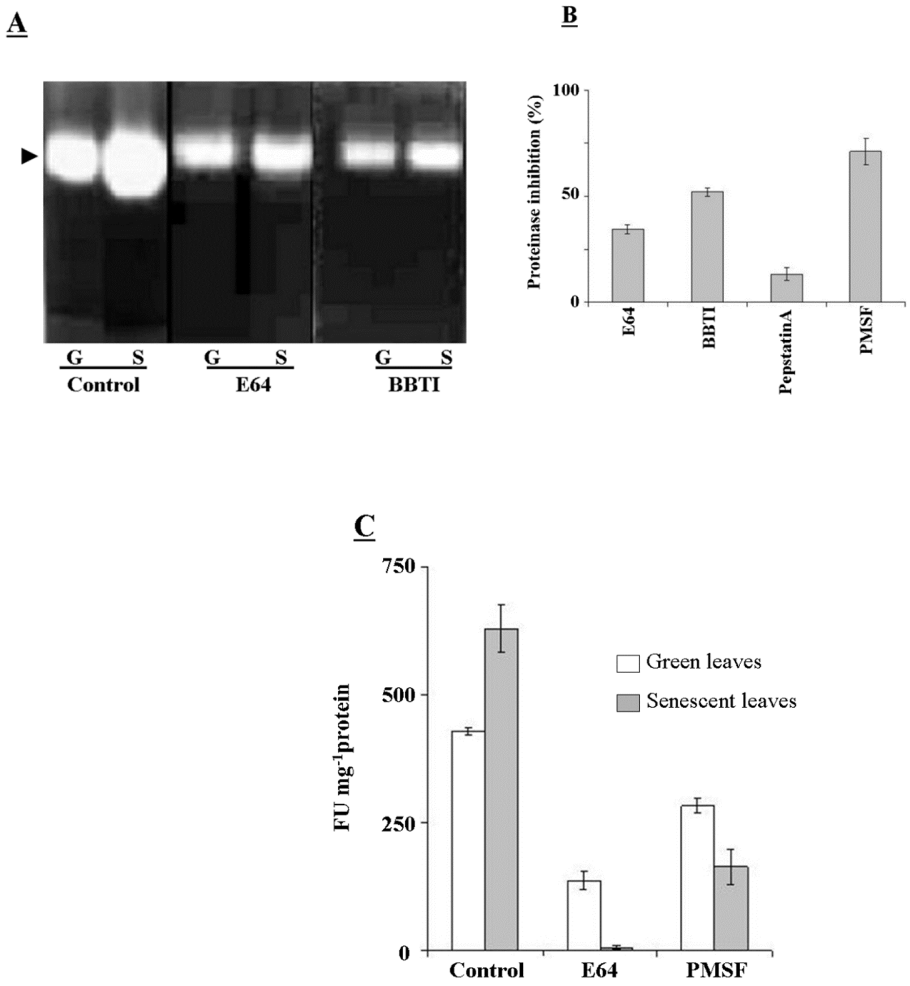


Figure 5.2 (A) Total proteinase activity in non-senescent, green (G) and senescent (S) tobacco leaves determined by activity gel electrophoresis on a mildly denaturing SDS-PAGE containing 0.1% gelatine and detection of proteinase activity after incubation with or without (Control) proteinases inhibitors. (B) Inhibition by different proteinase inhibitors of total proteinase activity measured by the azocasein test system and (C) of cysteine proteinase activity measured by a fluorescence assay. Data represent the means ± SD of four replicates.

Cysteine proteinase activity was also determined using Z-phe-arg-AMC (Figure 5.2C). These analyses showed that cysteine proteinase activity was significantly higher ($p \leq 0.05$) in senescent leaves (630±47 FU/mg protein) than mature green leaves (430±7 FU/mg protein). Proteinase activity in extracts of both types of leaves was significantly ($p \leq 0.05$) inhibited by E-64 (64% for green leaves and 99% for senescent leaves) or by PMSF (34% for green leaves and 74% for senescent leaves).

5.4.2 Isolation and analysis of *Nt*CP1 and *Nt*CP2

Two cDNA fragments with sizes of 514 bp and 507 bp were isolated from tobacco cDNAs that were synthesized from total RNA from senescent and mature green leaves, respectively. The isolated 514 bp cDNA fragment from senescent leaves had 86% similarity at the nucleotide level with the Petunia x hybrida cysteine proteinase, CP10 (GS Chaffin *et al*., unpublished, GenBank accession number AY662996). In comparison, the 507 bp cDNA fragment from non-senescent leaves had 83% similarity with Petunia x hybrida cysteine proteinase CP6 (GS Chaffin *et al*., unpublished, GenBank accession number AY662992). Applying the RACE technique and gene specific primers designed according to the sequence information obtained from the 514 bp and 507 bp fragments, a 1326 bp full-length cDNA sequence, denoted as *Nt*CP1, was obtained from the original 514 bp fragment derived from senescent leaves. This 1326 bp sequence of *Nt*CP1 had a 55 and 224 bp 5' and 3' un-translated regions (UTR), respectively, with an open reading frame (ORF) of 1050 bp (Figure 5.3A). Similarly, a full-length 1300 bp sequence, named *Nt*CP2, was obtained from the original 507 bp fragment derived from green, non-

senescent leaves and *Nt*CP2 had a 23 bp 5' and a 194 bp 3' UTR with an ORF of 1083 bp (Figure 5.3B).

A

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1       atccaagaaaagatttctatttctttttttcataataactagctagctgtttcataATGGCCTTTGCAAACCTTAGCCAAT
                                                          M  A  F  A  N  L  S  Q     8
81      ACCTTTGCTTAGCTTTGTTCTTCATATGTTTGGGACTTTGGAGCTCTCAAGTAGCTTTATCACGTCCAATAAACTATGAG
        Y  L  C  L  A  L  F  F  I  C  L  G  L  W  S  S  Q  V  A  L  S  R  P  I  N  Y  E    35
161     GCAACCATGCGTGCAAGGCATGACCAATGGATTGTACATCATGAAAAAGTTTACAAAGATTTGAACGAGAAAGAAGTGCG
         A  T  M  R  A  R  H  D  Q  W  I  V  H  H  E  K  V  Y  K  D  L  N  E  K  E  V  R   62
241     TTTCCAGATATTTAAAGAAAACGTGGAACGTATAGAAGCTTTTAACGCAGGTGAAGATAAAGGGTACAAACTCGGCTTTA
           F  Q  I  F  K  E  N  V  E  R  I  E  A  F  N  A  G  E  D  K  G  Y  K  L  G  F    88
321     ATAAATTTTCTGATCTCACGAATGAGGAATTTCGTGTATTACATACTGGTTACAAGAGGTCACACCCTAAGGTCATGACT
        N  K  F  S  D  L  T  N  E  E  F  R  V  L  H  T  G  Y  K  R  S  H  P  K  V  M  T   115
401     TCCTCAAAGGGAAAGACACATTTTAGGTACACTAATGTGACAGACATACCGCCAACTATGGATTGGAGAAAGAAAGGTGC
         S  S  K  G  K  T  H  F  R  Y  T  N  V  T  D  I  P  P  T  M  D  W  R  K  K  G  A  142
481     CGTCACCCCTATCAAGGACCAAAAGGAATGTGGGTGCTGTTGGGCATTTTCTGCAGTAGCTGCTATGGAAGGGCTACACC
           V  T  P  I  K  D  Q  K  E  C  G  C  C  W  A  F  S  A  V  A  A  M  E  G  L  H   168
561     AACTGAAAACAGGAGAGTTGATCCCTTTATCAGAGCAAGAGCTTGTAGACTGTGATGTCGAAGGCGAGGACGAAGGTTGC
        Q  L  K  T  G  E  L  I  P  L  S  E  Q  E  L  V  D  C  D  V  E  G  E  D  E  G  C   195
641     AGCGGTGGACTCTTGGACACTGCCTTTGATTTCATCCTGAAAAACAAGGGCCTCACAACAGAAGTAAACTATCCATACAA
         S  G  G  L  L  D  T  A  F  D  F  I  L  K  N  K  G  L  T  T  E  V  N  Y  P  Y  K  222
721     AGGAGAAGATGGTGTCTGCAACAAGAAAAAGTCAGCTCTTTCAGCAGCCAAAATTACAGGATATGAAGATGTGCCAGCGA
           G  E  D  G  V  C  N  K  K  K  S  A  L  S  A  A  K  I  T  G  Y  E  D  V  P  A   248
801     ACAGTGAGAAGGCTCTATTGCAGGCAGTGGCTAATCAACCTGTTTCGGTGGCAATAGACGGGAGTAGCTTCGATTTCCAG
        N  S  E  K  A  L  L  Q  A  V  A  N  Q  P  V  S  V  A  I  D  G  S  S  F  D  F  Q   275
881     TTCTATTCAAGTGGTGTATTCAGTGGATCATGCAGCACTTGGCTTAACCACGCTGTTACAGCAGTGGGATATGGTGCAAC
           F  Y  S  S  G  V  F  S  G  S  C  S  T  W  L  N  H  A  V  T  A  V  G  Y  G  A  T  302
961     AACTGACGGTACAAAGTATTGGATTATTAAGAATTCATGGGGCAGTAAATGGGGTGACAGTGGATATATGCGCATCAAAA
           T  D  G  T  K  Y  W  I  I  K  N  S  W  G  S  K  W  G  D  S  G  Y  M  R  I  K   328
1041    GGGATGTTCATGAGAAAGAAGGCCTTTGTGGACTTGCTATGGACGCTTCTTATCCCACTGCCTAAaaggaaattaattat
         R  D  V  H  E  K  E  G  L  C  G  L  A  M  D  A  S  Y  P  T  A                  349
1121    ccttagctttttttcagtacttttcaaccatgtttatatatgtgaatattgtgttgtgggtttgaggcagacttggattag
1201    actgtccatacatagttaaagcacttaaaaggtgccagcttttaaattgtaattttattttcatcattgtgaacttaata
1281    atggttattgtctaaatttgatttgactaaaaaaaaaaaaaaaaaa
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B

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1       atcttggagtctgagtctttaaaATGAAGAAGTTATTTCTGGTTCTTTTCTCTTTGGCTTTGGTACTTAGGCTTGGGGAG
                              M  K  K  L  F  L  V  L  F  S  L  A  L  V  L  R  L  G  E   19
81      AGTTTCGATTTCCACGAGAAGGAGTTGGAAACTGAGGAAAAATTGTGGGAGTTGTATGAGAGATGGAGAAGCCATCACAC
         S  F  D  F  H  E  K  E  L  E  T  E  E  K  L  W  E  L  Y  E  R  W  R  S  H  H  T   46
161     TGTATCGAGGAGCCTTGATGAGAAAGACAAGAGGTTCAATGTGTTCAAGGCTAATGTACACTATGTTCACAACTTCAACA
          V  S  R  S  L  D  E  K  D  K  R  F  N  V  F  K  A  N  V  H  Y  V  H  N  F  N   72
241     AGAAGGATAAGCCTTATAAGTTGAAATTGAACAAGTTTGCAGACATGACTAACCATGAATTCAGACACCATTATGCTGGT
         K  K  D  K  P  Y  K  L  K  L  N  K  F  A  D  M  T  N  H  E  F  R  H  H  Y  A  G   99
321     TCCAAGATTAAGCATCATCGTTCTTTTCTTGGAGCTTCACGAGCAAATGGAACTTTCATGTACGCCAATGTGGAAGATGT
          S  K  I  K  H  H  R  S  F  L  G  A  S  R  A  N  G  T  F  M  Y  A  N  V  E  D  V  126
401     CCCTCCCTCTGTTGACTGGAGGAAGAAGGGTGCTGTTACTCCTGTCAAAGACCAAGGCAAATGTGGAAGTTGCTGGGCAT
          P  P  S  V  D  W  R  K  K  G  A  V  T  P  V  K  D  Q  G  K  C  G  S  C  W  A   152
481     TTTCAACTGTCGTTGCAGTAGAGGGGATAAACCAAATCAAAACAAATGAGTTAGTATCTTTATCAGAACAAGAACTTGTT
         F  S  T  V  V  A  V  E  G  I  N  Q  I  K  T  N  E  L  V  S  L  S  E  Q  E  L  V   179
561     GACTGTGACACTAGTCAAAACCAAGGATGCAATGGAGGGTTGATGGACATGGCATTTGAATTCATCAAGAAGAAGGGAGG
          D  C  D  T  S  Q  N  Q  G  C  N  G  G  L  M  D  M  A  F  E  F  I  K  K  K  G  G  206
641     CATCAATACTGAAGAGAACTATCCATACATGGCTGAAGGTGGCGAGTGTGATATTCAAAAGAGGAATTCTCCTGTGGTAT
          I  N  T  E  E  N  Y  P  Y  M  A  E  G  G  E  C  D  I  Q  K  R  N  S  P  V  V   232
721     CAATTGACGGATATGAGGATGTTCCTCCTAATGATGAGGATTCCCTACTTAAAGCAGTAGCCAACCAGCCTGTTTCTGTA
         S  I  D  G  Y  E  D  V  P  P  N  D  E  D  S  L  L  K  A  V  A  N  Q  P  V  S  V   259
801     GCTATACAAGCTTCAGGTTCTGACTTCCAGTTCTACTCTGAGGGTGTATTCACCGGAGACTGTGGTACTGAGTTGGACCA
          A  I  Q  A  S  G  S  D  F  Q  F  Y  S  E  G  V  F  T  G  D  C  G  T  E  L  D  H  286
881     TGGTGTGGCAATTGTGGGCTATGGCACAACCCTTGATGGAACCAAATACTGGATTGTGAGGAACTCGTGGGGACCTGAAT
          G  V  A  I  V  G  Y  G  T  T  L  D  G  T  K  Y  W  I  V  R  N  S  W  G  P  E   312
961     GGGGAGAAAAAGGATACATTAGGATGCAACGCGAGATTGATGCTGAAGAGGGATTGTGTGGTATAGCAATGCAACCATCC
         W  G  E  K  G  Y  I  R  M  Q  R  E  I  D  A  E  E  G  L  C  G  I  A  M  Q  P  S   339
1041    TACCCCATCAAGACTTCATCAAGCAACCCCACAGGGTCTCCTGCAACAGCACCTAAGGATGAACTCtaagttacatatta
         Y  P  I  K  T  S  S  S  N  P  T  G  S  P  A  T  A  P  K  D  E  L                  361
1121    tccactcagcctcatttagtgtctgttttagtttctgtgcaagtaataggcaactacccataagaatgtgtaacataaga
1201    tgcatttagtttcatggtgtcgcattataatgttctgtaaactgcattggaatatgattcagtgaaacatttcctttcct
        gcaaaaaaaaaaaaaaaaaa
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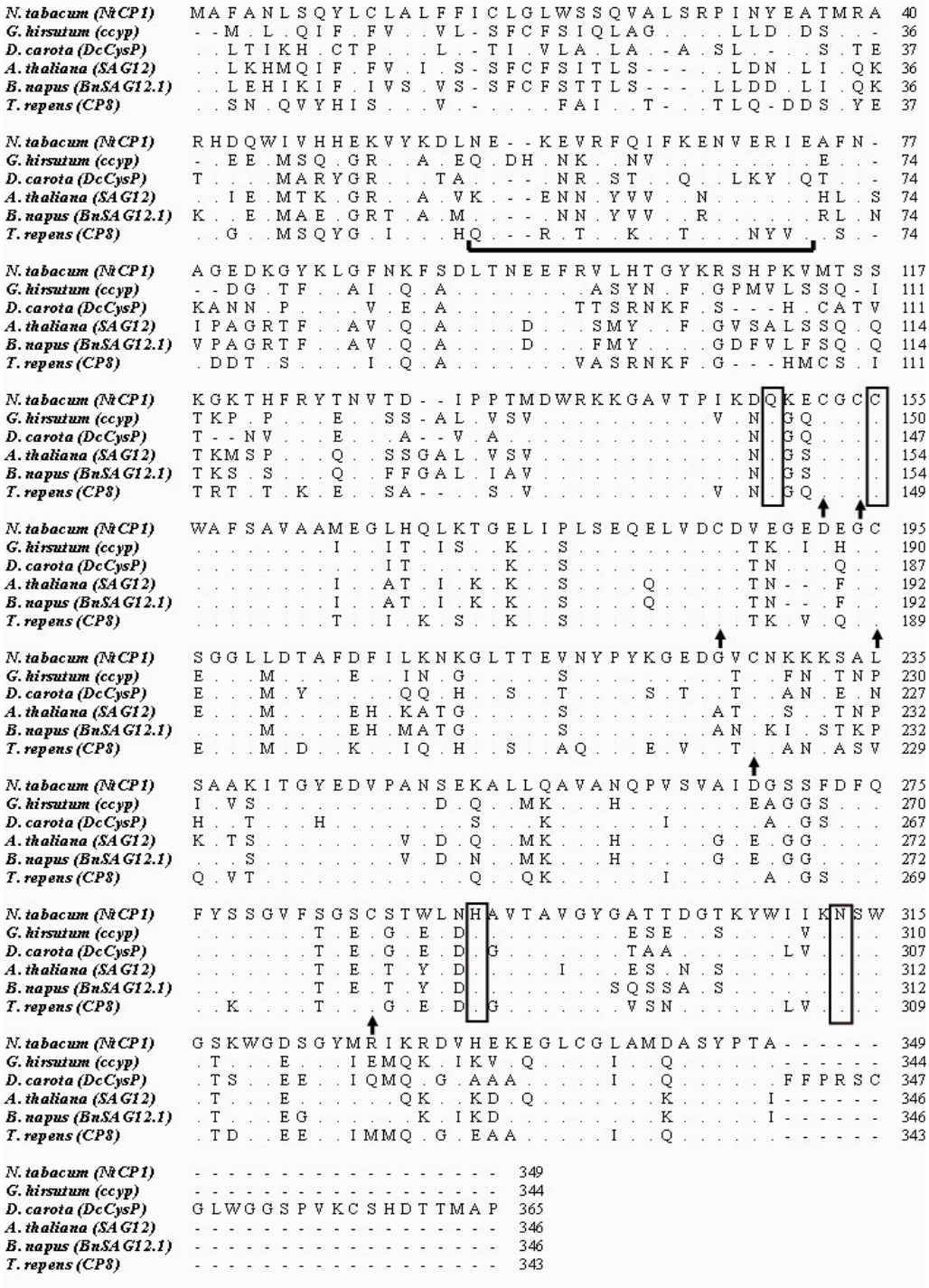
Figure 5.3 Nucleotide and deduced amino acid sequence of *Nt*CP1 (A) and *Nt*CP2 (B). The catalytic triad Cys, His and Asn and also the Glu active site residue are circled. The GCNGG motif is double-underlined. The 5' and 3'-untranslated regions are shown in lower case and signal peptides M1-S29 for *Nt*CP1 and M1-S20 for *Nt*CP2 are showm in bold face with hydrophobic cores underlined. ERFNIN motif E58-N77 for *Nt*CP1 and E53-N72 for *Nt*CP2 is shown in rectangular box and the KDEL (K358-L361) motif of *Nt*CP2 is single underlined. Numbers on the left and right margines represent nucleotide and deduced amino acid sequences, respectively. GenBank accession numbers of the *Nt*CP1 and *Nt*CP2 sequnce are AY881011 and AY881010, respectively.

A predicted co-translational N-terminal signal peptide with a hydrophobic core was identified in the deduced amino acid sequences of *Nt*CP1 and *Nt*CP2 (Figures 5.3 A and B). Such signal peptide carrying hydrophobic residues are typical of sequences allowing endoplasmic reticulum (ER) targeting. Further, the pro-domains of both *Nt*CP1 and *Nt*CP2 (Figure 5.3 A and B) contains a conserved non-contiguous ERFNIN motif ($EX_3RX_3FX_2NX_3I/VX_3N$) typical for cysteine proteinases in the Cathepsin L and H like proteinases (Karrer *et al*., 1993). A GCNGG motif was identified in both *Nt*CP2 (G188-G192; Figure 5.3B) and *Nt*CP1 (Figure 5.3A; G194-G198). With the exception of the central Asn (N) residue, this GCNGG motif is invariant in all ERFNIN proteinases and also in the cathepsin B-like proteinases (Karrer *et al*., 1993). In papain-like proteinases the Cys residue in the GCNGG motif is involved in the formation of a disulfide bridge. In addition, *Nt*CP2 has a C-terminus KDEL motif (Figure 5.3B) which is absent from

*Nt*CP1. Both *Nt*CP1 and *Nt*CP2 contain the conserved cysteine proteinase catalytic triade Cys, His and Asn as well as the conserved Glu residue (Figures 5.3 and 5.4).

The *Nt*CP1 and *Nt*CP2 are 52% and 50% identical when the nucleotide and the amino acid sequences, were compared, respectively. Using a BLASTN and BLASTP search, both *Nt*CP1 and *Nt*CP2 were identified to have ab high homology to the group of papain-like cysteine proteinases. Alignment of *Nt*CP1 derived from senescent leaves against already reported cysteine proteinase amino acid sequences in the NCBI database revealed a similarity to several papain-like cysteine proteinases (Figure 5.4A). This includes a 57-58% identity with *Dacus carota* DcCysP2 (Mitsuhashi *et al*., 2004), *Ipomea batatas* SPG31 (Chen *et al*., 2002), *Arabidopsis thaliana* SAG12 (Lohman *et al*., 1994), and *Brassica napus* SAG12-1 (Noh and Amasino, 1999). *Nt*CP1 is less related to other tobacco cysteine proteinases so far reported. This includs CPR1 from germinating tobacco seeds (55% similarity; GenBank accession number Z99173), drought-inducible CPR2 (39%; GenBank accession number AJ242994), NTCP-23 (40%; Ueda *et al*., 2000) and wound-inducible CYP-7 (39%; Linthorst *et al*., 1993) and CYP-8 (39%; Linthorst *et al*., 1993).

A


N. tabacum (NtCP1) MAFANLSQYLCLALFFICLGLWSSQVALSRPINYEATMRA 40
G. hirsutum (ccyp) --M.L.QIF.FV..VL-SFCFSIQLAG....LLD.DS...- 36
D. carota (DcCysP) ..LTIKH.CTP...L..TI.VLA.LA..A.SL....S.TE 37
A. thaliana (SAG12) ..LKHMQIF.FV.I.S-SFCFSITLS-----LDN.LI.QK 36
B. napus (BnSAG12.1) ..LEHIKIF.IVS.VS-SFCFSTTLS----LLDD.LI.QK 36
T. repens (CP8) ..SN.QVYHIS...V.-.....FAI..T-..TLQ-DDS.YE 37
N. tabacum (NtCP1) RHDQWIVHHEKVYKDLNE--KEVRFQIFKENVERIEAFN- 77
G. hirsutum (ccyp) --EE.MSQ.GR..A.EQ.DH.NK..NV.........E...- 74
D. carota (DcCysP) T....MARYGR...TA.----NR.ST..Q..LKY.QT...- 74
A. thaliana (SAG12) ..IE.MTK.GR..A.VK---ENN.YVV..N.....HL.S 74
B. napus (BnSAG12.1) K..E.MAE.GRT.A.M.----NN.YVV..R.....RL.N 74
T. repens (CP8) ..G..MSQYG.I...HQ---R.T..K..T...NYV..S..- 74
N. tabacum (NtCP1) AGEDKGYKLGFNKFSDLTNEEFRVLHTGYKRSHPKVMTSS 117
G. hirsutum (ccyp) --DG.TF..AI.Q.A.......ASYN.F.GPMVLSSQ-I 111
D. carota (DcCysP) KANN.P....V.E.A.....TTSRNKF.S---H.CATV 111
A. thaliana (SAG12) IPAGRTF..AV.Q.A....D...SMY..F.GVSALSSQ.Q 114
B. napus (BnSAG12.1) VPAGRTF..AV.Q.A....D...FMY....GDFVLFSQ.Q 114
T. repens (CP8) .DDT.S....I.Q.A.......VASRNKF.G---HMCS.I 111
N. tabacum (NtCP1) KGKTHFRYTNVTD--IPPTMDWRKKGAVTPIKDQKECGCC 155
G. hirsutum (ccyp) TKP.P...E..SS-AL.VSV.........V.N.GQ....150
D. carota (DcCysP) T--NV...E...A--V.A.............N.GQ....147
A. thaliana (SAG12) TKMSP...Q..SSGAL.VSV.........N.GS....154
B. napus (BnSAG12.1) TKS.S...Q..FFGAL.IAV.........N.GS....154
T. repens (CP8) TRT.T.K.E..SA----S.V.........V.N.GQ....149
N. tabacum (NtCP1) WAFSAVAAMEGLHQLKTGELIPLSEQELVDCDVEGEDEGC 195
G. hirsutum (ccyp) .........I..IT.IS..K..S.........TK.I.H... 190
D. carota (DcCysP) .........IT.....K..S.........TN...Q... 187
A. thaliana (SAG12) .........I..AT.I.K.K..S....Q.....TN--.F... 192
B. napus (BnSAG12.1) .........I..AT.I.K.K..S....Q.....TN--.F... 192
T. repens (CP8) .........T..I.K.S..K..S.........TK.V.Q... 189
N. tabacum (NtCP1) SGGLLDTAFDFILKNKGLTTEVNYPYKGEDGVCNKKKSAL 235
G. hirsutum (ccyp) E...M....E..IN.G.....S.........T..FN.TNP 230
D. carota (DcCysP) E...M.Y.....QQ.H..S..T.....S.T..T..AN.E.N 227
A. thaliana (SAG12) E...M....EH.KATG.....S.........AT..S..TNP 232
B. napus (BnSAG12.1) ....M....EH.MATG.....S.........AN.KI.STKP 232
T. repens (CP8) E...M.D..K..IQ.H..S..AQ...E.V..T..AN.ASV 229
N. tabacum (NtCP1) SAAKITGYEDVPANSEKALLQAVANQPVSVAIDGSSFDFQ 275
G. hirsutum (ccyp) I.VS.........D.Q..MK...H.........EAGGS... 270
D. carota (DcCysP) H..T...H.....S...K.....I.....A.GS... 267
A. thaliana (SAG12) K.TS.....V.D.Q..MK...H.....G.E.GG.... 272
B. napus (BnSAG12.1) ...S.....V.D.N..MK...H.....G.E.GG.... 272
T. repens (CP8) Q.VT.....Q..QK.....I.....A.GS... 269
N. tabacum (NtCP1) FYSSGVFSGSCSTWLNHAVTAVGYGATTDGTKYWIIKNSW 315
G. hirsutum (ccyp) .....T.E.G.E.D.....ESE..S....V.... 310
D. carota (DcCysP) .....T.E.G.E.D.G.....TAA.....LV.... 307
A. thaliana (SAG12) .....T.E.T.Y.D.....I...ES.N.S..... 312
B. napus (BnSAG12.1) .....T.E.T.Y.D.....SQSSA.S..... 312
T. repens (CP8) ..K....T...G.E.D.G.....VSN.....LV.... 309
N. tabacum (NtCP1) GSKWGDSGYMRIKRDVHEKEGLCGLAMDASYPTA------ 349
G. hirsutum (ccyp) .T...E...IEMQK.IKV.Q....I..Q.......------ 344
D. carota (DcCysP) .TS..EE..IQMQ.G.AAA....I..Q.....FFPRSC 347
A. thaliana (SAG12) .T...E.....QK..KD.Q.....K.....I------ 346
B. napus (BnSAG12.1) .T...EG.....K.IKD.....K.....I------ 346
T. repens (CP8) .TD..EE..IMMQ.G.EAA....I..Q.....------ 343
N. tabacum (NtCP1) ---------- 349
G. hirsutum (ccyp) ---------- 344
D. carota (DcCysP) GLWGGSPVKCSHDTTMAP 365
A. thaliana (SAG12) ---------- 346
B. napus (BnSAG12.1) ---------- 346
T. repens (CP8) ---------- 343

B

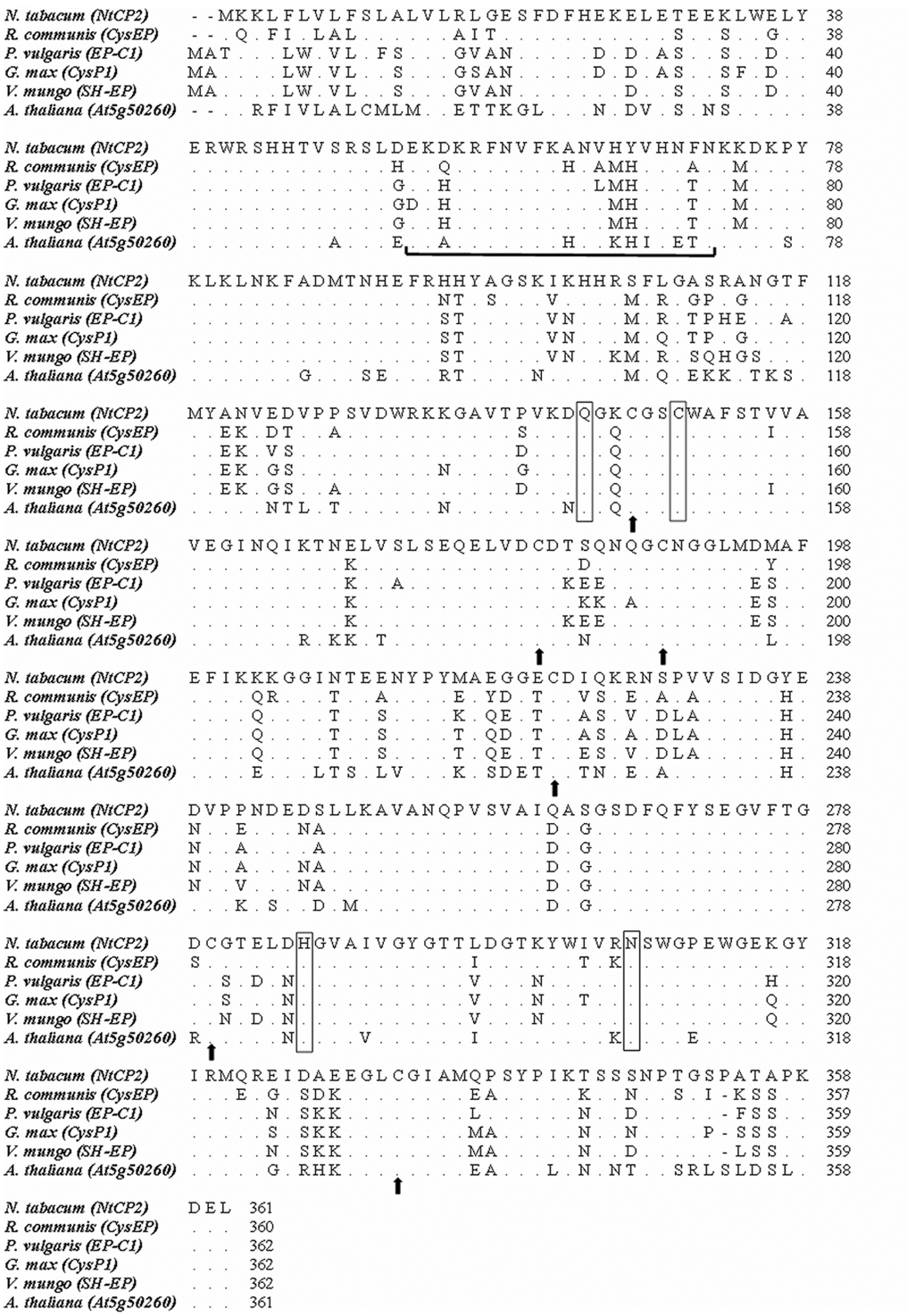

N. tabacum (NtCP2) - -MKKLFLVLFSLALVLRLGESFDFHEKELETEEKLWELY 38
R. communis (CysEP) - - - Q . FI . LAL . . . . . AIT . . . . . S . . S . . G . . 38
P. vulgaris (EP-C1) MAT . . . LW . VL . FS . . . GVAN . . . . . D . D . AS . . S . . D . . 40
G. max (CysP1) MA . . . . LW . VL . . S . . . GSAN . . . . . D . D . AS . . SF . D . . 40
V. mungo (SH-EP) MA . . . . LW . VL . . S . . . GVAN . . . . . D . . S . . S . . D . . 40
A. thaliana (At5g50260) - - - - RFIVLALCMLM . . ETTKGL . . . N . DV . S . NS 38
N. tabacum (NtCP2) ERWRSHHTVSRSLDEKDKRFNVFKANVHYVHNFNKKDKPY 78
R. communis (CysEP) . . . . . H . . Q . . . . . H . AMH . . . A . . M 78
P. vulgaris (EP-C1) . . . . . G . . H . . . . . LMH . . . T . . M 80
G. max (CysP1) . . . . . GD . H . . . . . MH . . . T . . M 80
V. mungo (SH-EP) . . . . . G . . H . . . . . MH . . . T . . M 80
A. thaliana (At5g50260) . . . . . A . . . E . . A . . . . . H . . KHI . ET . . . . . S . 78
N. tabacum (NtCP2) KLKLNKFADMTNHEFRHHYAGSKIKHHRSFLGASRANGTF 118
R. communis (CysEP) . . . . . NT . S . . . V . . . . M . R . GP . G 118
P. vulgaris (EP-C1) . . . . . ST . . . . VN . . . M . R . TPHE . . A . 120
G. max (CysP1) . . . . . ST . . . . VN . . . M . Q . TP . G 120
V. mungo (SH-EP) . . . . . ST . . . . VN . . KM . R . SQHGS . . . 120
A. thaliana (At5g50260) . . . . . G . . . SE . . . RT . . . . N . . . . . M . Q . EKK . TKS . 118
N. tabacum (NtCP2) MYANVEDVPPSVDWRKKGAVTPVKDQGKCGSCWAFSTVVA 158
R. communis (CysEP) . . EK . DT . . A . . . . . S . . . . Q . . . . . I . . 158
P. vulgaris (EP-C1) . . EK . VS . . . . . D . . . . Q 160
G. max (CysP1) . . EK . GS . . . . . N . . . G . . . . Q 160
V. mungo (SH-EP) . . EK . GS . . A . . . . . D . . . . Q . . . . . I . . 160
A. thaliana (At5g50260) . . . . NTL . T . . . . . N . . . . . N . . Q 158
N. tabacum (NtCP2) VEGINQIKTNELVSLSEQELVDCDTSQNQGCNGGLMDMAF 198
R. communis (CysEP) . . . . . K . . . . . D . . . . . Y . . 198
P. vulgaris (EP-C1) . . . . . K . . A . . . . . KEE . . . . . ES . . 200
G. max (CysP1) . . . . . K . . . . . KK . A . . . . . ES . . 200
V. mungo (SH-EP) . . . . . K . . . . . KEE . . . . . ES . . 200
A. thaliana (At5g50260) . . . . . R . KK . T . . . . . N . . . . . L . . 198
N. tabacum (NtCP2) EFIKKKGGINTEENYPYMAEGGECDIQKRNSPVVSIDGYE 238
R. communis (CysEP) . . . . QR . . . T . . A . . . E . YD . T . . VS . E . A . A . . . . H . 238
P. vulgaris (EP-C1) . . . . Q . . . . T . . S . . . . K . QE . T . . AS . V . DLA . . . . H . 240
G. max (CysP1) . . . . Q . . . . T . . S . . . . T . QD . T . . AS . A . DLA . . . . H . 240
V. mungo (SH-EP) . . . . Q . . . . T . . S . . . . T . QE . T . . ES . V . DLA . . . . H . 240
A. thaliana (At5g50260) . . . . E . . . LTS . LV . . . K . SDET . . TN . E . A . . . . . H . 238
N. tabacum (NtCP2) DVPPNDEDSLLKAVANQPVSVAIQASGSDFQFYSEGVFTG 278
R. communis (CysEP) N . . E . . . NA . . . . . D . G 278
P. vulgaris (EP-C1) N . . A . . . . A . . . . . D . G 280
G. max (CysP1) N . . A . . . NA . . . . . D . G 280
V. mungo (SH-EP) N . . V . . . NA . . . . . D . G 280
A. thaliana (At5g50260) . . . K . S . . D . M . . . . . D . G 278
N. tabacum (NtCP2) DCGTELDHGVAIVGYGTTLDGTKYWIVRNSWGPEWGEKGY 318
R. communis (CysEP) S . . . . . I . . . . . T . K 318
P. vulgaris (EP-C1) . . S . D . N . . . . . V . . N . . . . . H . . 320
G. max (CysP1) . . S . . . N . . . . . V . . N . . T . . . . . Q . . 320
V. mungo (SH-EP) . . N . D . N . . . . . V . . N . . . . . Q . . 320
A. thaliana (At5g50260) R . . . . . N . . . . V . . . . . I . . . . . K . . . . E 318
N. tabacum (NtCP2) IRMQREIDAEEGLCGIAMQPSYPIKTSSSNPTGSPATAPK 358
R. communis (CysEP) . . . E . G . SDK . . . . . EA . . . . . K . . N . . S . I - KSS . . 357
P. vulgaris (EP-C1) . . . . . N . SKK . . . . . L . . . . . N . . D . . . . . - FSS . . 359
G. max (CysP1) . . . . . S . SKK . . . . . MA . . . . . N . . N . . . . P - SSS . . 359
V. mungo (SH-EP) . . . . . N . SKK . . . . . MA . . . . . N . . D . . . . . - LSS . . 359
A. thaliana (At5g50260) . . . . . G . RHK . . . . . EA . . . L . N . NT . . SRLSLDSL . 358
N. tabacum (NtCP2) DEL 361
R. communis (CysEP) . . . 360
P. vulgaris (EP-C1) . . . 362
G. max (CysP1) . . . 362
V. mungo (SH-EP) . . . 362
A. thaliana (At5g50260) . . . 361

Figure 5.4 Multiple alignment of *Nt*CP1 with related senescence-associated proteinases (A) and *Nt*CP2 with related KDEL-tailed proteinases (B). Amino acid sequences were aligned by Clustal W multiple alignment software. Identical amino acids are represented with dots. Boxed amino acids indicate conserved amino acid residues involved in proteinase activity. Filled arrows indicate a predicted cysteine residues involved in disulfide bridge formation. Conserved ERFNIN motif is underlined (B).

In comparison, the similarity search using *Nt*CP2 derived from mature green leaves revealed an amino acid similarity (68-72%) with KDEL-tailed plant cysteine proteinases (Figure 5.4B). This includs similarity to *Ricinus cumunis* Cys-EP (Schmid *et al*., 1998), *Phaseolus vulgaris* EP-C1 (Tanaka *et al*., 1991), *Glycine max* CysP1 (Ling *et al*., 2003) and *Vigna mungo* sulfhydryl-endopeptidase (SH-EP; Akasofu *et al*., 1989). *Nt*CP2 has, however, a much lower similarity to other already identified *Nicotiana* cysteine proteinases. On the amino acid level this includes a 31% similarity with tobacco NTCP-23, a 30% and 31% similarity with CYP-7 and CYP-8, a 30% similarity with CPR2 and 49% similarity with CPR1. Database searches also revealed that *Nt*CP2 has a very high (94%) similarity with a partial N-terminal sequence for a cysteine proteinase derived from tobacco anthers (TP Beals and RB Goldberg, unpublished; GenBank accession number U57824). However none of the database tobacco sequences have such a well-defined KDEL tail as *Nt*CP2.

A phylogenetic tree, constructed to identify the relatedness of amino acid sequences of *Nt*CP1 and *Nt*CP2 to other members of the papain-like cysteine proteinase sub-family

(Figure 5.5) revealed that the two sequences are localised in two separate groups. *Nt*CP1 is grouped in group 4 or C1A-4 according to Beers *et al*. (2004). This group includes leaf senescence-specific proteinases, such as *Arabidopsis* SAG12, *Brassica napus* SAG12-1 and *Ipomea batatas* SPG31. In contrast, *Nt*CP2 is located in group 2. This group contains KDEL-tailed family members, such as *Ricinus cummunis* Cys-EP and *Vigna mungo* SH-EP.

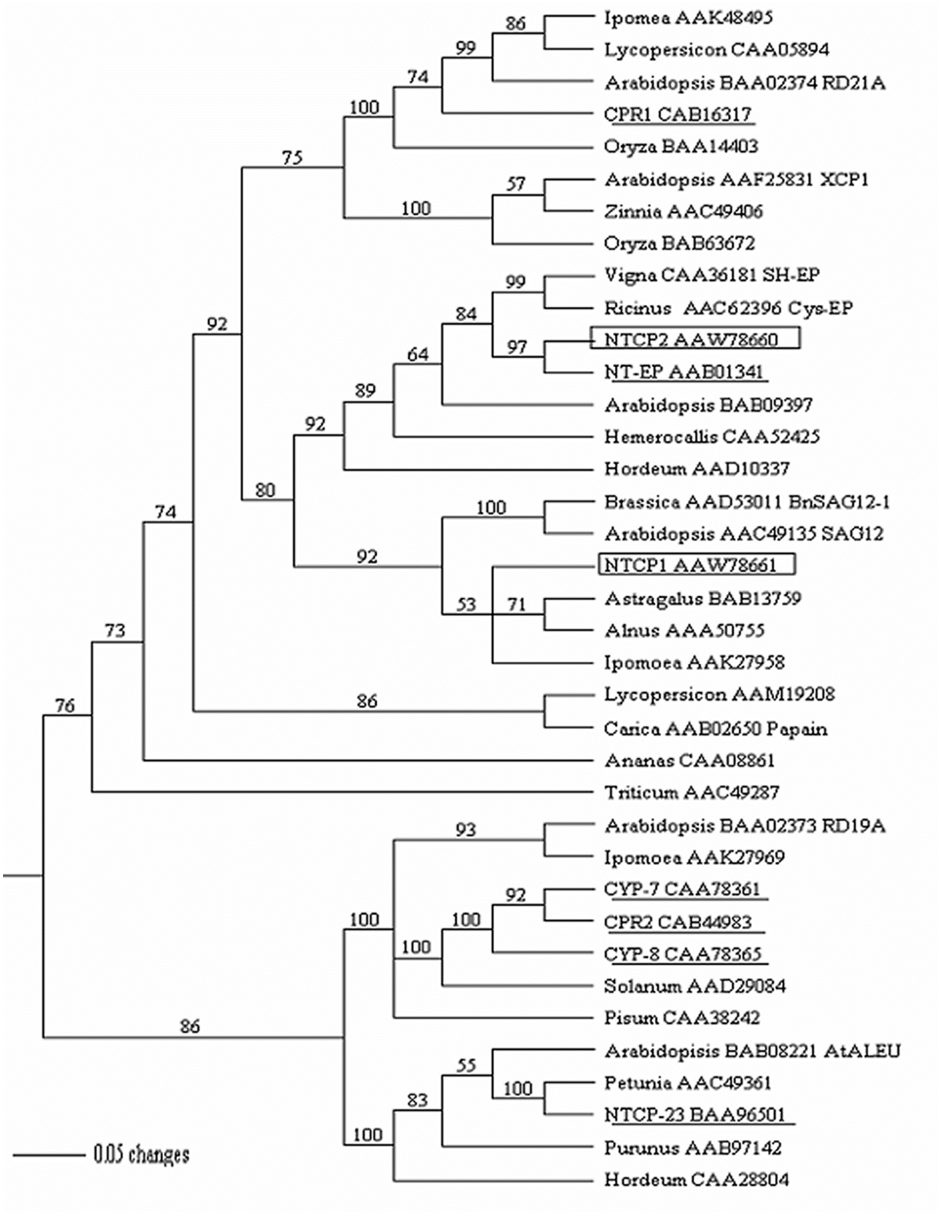


Figure 5.5 Phylogenetic tree of plant papain-like cysteine proteinases. Tobacco papain-like cysteine proteinases are underlined. Genus name and accession numbers are shown including gene name for reported proteinases. *Nt*CP1 and *Nt*CP2 are boxed. Proteinases with full pre-propetides were used for both alignment and generation of tree with the exception of NT-EP like proteinases.

5.4.3 Copy number and expression of *Nt*CP1 and *Nt*CP2

Southern blot analysis of isolated tobacco genomic DNA showed that multiple copies of *Nt*CP1 and *Nt*CP2 exist in the tobacco genome (Figure 5.6). Two distinct hybridization products and two overlapping products were found with labelled *Nt*CP1 and genomic DNA digested with *Xba*I and *Eco*RI, respectively. In contrast, four hybridization products were found with labelled *Nt*CP2 and *Xba*I. More than six products were found with *Eco*RI-digested genomic DNA.

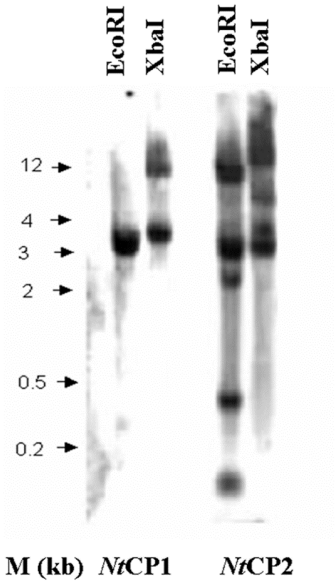


Figure 5.6 Southern blot analysis of *Nt*CP1 and *Nt*CP2. For analysis, genomic DNA from tobacco plants was digested with indicated restriction enzymes and probed with gene specific probes after blotting onto a membrane. Probes used for detection were labelled PCR products derived from cloned *Nt*CP1 and *Nt*CP2 genomic DNA fragments.

Northern blot analysis revealed that *Nt*CP1 was present in senescent leaves. In contrast, no *Nt*CP1 transcripts were detected in mature green leaves. *Nt*CP2 transcripts were detected in mature green leaves (Figure 5.7A) but not in senescent leaves. *Nt*CP1 expression was not induced in mature green leaves by exposure to abiotic stress in the present study, including drought, heat and a combination of both stresses (Figure 5.7B). *Nt*CP2 transcripts were much decreased in mature green leaves following drought treatment and transcript could not be detected following heat, drought or combination of both stress (Figure 5.7B).

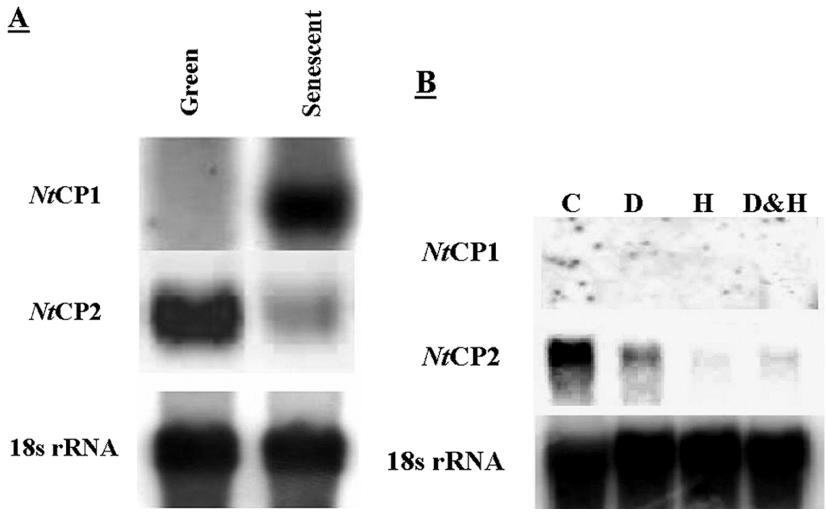


Figure 5.7 (A) Northern blot analysis for detection of *Nt*CP1 and *Nt*CP2 expression in mature green and senescent leaves. (B) *Nt*CP1 and *Nt*CP2 transcripts were measured in leaves exposed to drought (D), heat (H) or a combination of drought and heat stress (D & H). C represents RNA from untreated mature green leaves. Total RNA was hybridised with labelled PCR products derived from *Nt*CP1 and *Nt*CP2 cloned fragments. To ensure equal sample abundance on gels 18S rRNA was used to monitor loading equivalence.

5.5 Discussion

5.5.1 *Nt*CP1 and *Nt*CP2 sequence analysis

In the present study we have identified two novel tobacco coding sequences encoding cysteine proteinases, denoted as *Nt*CP1 and *Nt*CP2. According to their deduced amino acid sequences both proteinases can be classified as belonging to the sub-family C1A of papain-like cysteine proteinases (MEROPS peptidase database, http://merops.sanger.ac.uk, Rawlings *et al*., 2004). Based on nucleotide as well as amino acid analysis, *Nt*CP2 is phylogenetically distant from *Nt*CP1. Except for a partial N-terminal sequence from tobacco anthers, which is related to *Nt*CP2, the data presented here provide evidence that these are novel coding sequences that are only distantly related to each and other tobacco cysteine proteinases reported to date.

*Nt*CP2 is (to my knowledge) the first KDEL-motif-containing cysteine proteinase to be isolated from tobacco. No KDEL motif was found in *Nt*PC1. Papain-like cysteine proteinases with a KDEL motif are involved in programmed cell death (Guerrero *et al*., 1998; Schmid *et al*., 1998; Gietl and Schmid, 2001 and Ling *et al*., 2003). Since *Nt*CP2 belongs to the KDEL cysteine proteinase group, it might also play a role in programmed cell death. There is also considerable evidence that KDEL proteinases accumulate in cell vesicles, such as ER-derived ricinosomes (Schmid *et al*., 2001) or in KDEL vesicles (Toyooka *et al*., 2000), before being transported to the vacuoles. Okamoto *et al*. (2003) suggested that the KDEL motif of KDEL proteinases could act as enhancers for vacuolar transport, because the KDEL motif appears to be directly involved in the formation of KDEL vesicles and vacuole transportation. Transformed Arabidopsis plants expressing a

mutant SH-EP proteinase lacking the KDEL motif were unable to develop KDEL vesicles, the mutant SH-EP being mainly secreted into the intercellular spaces of the transformed plants, which showed abnormal development and accelerated death (Okamoto *et al*., 2003).

5.5.2 *Nt*CP1 characterization

Senescent tobacco leaves expressing *Nt*CP1 had increased proteinase activity. The inhibitor studies showed that at least three classes of proteinase (cysteine, serine and aspartic proteinases) are present in senescent tobacco leaves. Moreover, increased proteinase activity was accompanied by decreases in chlorophyll and protein content. *Nt*CP1 transcripts were only detectable in senescent tobacco leaves. Therefore, it would appear that *Nt*CP1 is not expressed in mature green leaves. Similarly, *Nt*CP1 transcripts were not detected in mature green leaves following exposure to drought or heat or a combination of both stresses. *Nt*CP1 was only expressed in senescent leaves, a result comparable with that obtained for another cysteine proteinase, SAG12. This proteinase, previously identified by Lohman *et al*. (1994) in *Arabidopsis*, exhibits one of the highest levels of induction during *Arabidopsis* leaf senescence. It is also often used as a senescence marker in studies in plant development as it not induced by stress-induced programmed cell death, unlike other types of SAG genes (Brodersen *et al*., 2002). Phylogenetic analysis revealed that *Nt*CP1 clusters with SAG12 and also with BnSAG12-1 (Noh and Amasino, 1999). Since both *Arabidopsis* SAG12 and *Brassica* BnSAG12-1 are considered to be senescence-specific genes these data support the notion that *Nt*CP1 is a developmental marker for tobacco leaf senescence (Lohman *et al*., 1994; Noh and

Amasino, 1999; Wan *et al*., 2002). Hence, *Nt*CP1 could be a good marker for developmental senescence in tobacco as it is not induced in mature green leaves in optimal or stress conditions.

Like SAG12, *Nt*CP1 belongs to class I type SAGs. In general, SAGs can be separated into two classes based on their temporal gene expression pattern during leaf senescence (Gan and Amasino, 1997). Class I type SAGs are expressed only during leaf senescence. Therefore, they are denoted as being senescence-specific. In contrast, class II type SAGs have a low basal expression throughout leaf development but at the onset of senescence their expression is significantly enhanced. Unlike *Nt*CP1, the tobacco NTCP-23 cysteine proteinase sequence shows a typical class II type SAG expression pattern (Ueda *et al*., 2000).

A recent study also showed that SAG12 and possibly other senescence-associated cysteine proteinases are restricted to senescence-associated vacuoles (SAVs; Otegui *et al*., 2005). These SAVs are more acidic than the central vacuoles. The specific development of SAV in cells containing chloroplasts might indicate the possible involvement of SAVs in degradation of chloroplast proteins (Otegui *et al*., 2005). While the exact cellular localization of *Nt*CP1 is unknown, Rubisco degradation is prevented in tobacco leaves expressing a rice cysteine proteinase inhibitor (OC-I). This suggests that cysteine proteinases are important for degradation of chloroplast proteins such as Rubisco.

5.5.3 *Nt*C2 characterization

*Nt*CP2 transcripts were detected only in mature green leaves. Moreover, *Nt*CP2 transcripts were significantly decreased in leaves following exposure to drought or heat stress or a combination of both stresses. Such drought-induced down-regulation of cysteine proteinase expression has also been reported for other cysteine proteinases (Weaver *et al*., 1998). The *Nt*CP2 clusters with a group of cysteine proteinases whose expression profiles are rather variable between species. For example, SEN102 and SEN11 transcripts from *Hemerocallis* spp flowers (Valpuesta *et al.*, 1995; Guerrero *et al*., 1998) that are closely related to *Nt*CP2 accumulate at high level in senescing flowers, but in leaves higher level of accumulation was found in green leaves and their expression was lower in senescing leaves. Moreover, two KDEL-tailed cysteine proteinases also belonging to this group CysP1 and CysP2 have been found in senescent soybean cotyledons, flowers, roots and pods, as well as in young leaves (Ling *et al*., 2003). In tobacco, *Nt*CP2 expression was found to be higher in mature green leaves and significantly decreased in senescent leaves. This perhaps suggests that different species use either similar or identical gene-products to modulate the development of different organs including leaves.

In conclusion, two novel tobacco leaf cysteine proteinases have been identified, which are differentially expressed during development and in response to stress. While to date no information concerning their cellular function or localization is available, the data presented here indicate different roles in leaf development, since *Nt*CP1 is also only

expressed in senescent tobacco leaves *Nt*CP1, like SAG12, can be used as a specific molecular marker for age mediated leaf senescence in tobacco.

CHAPTER SIX

SUMMARY AND PERSPECTIVE

Environmental abiotic and biotic stresses are worldwide the primary limitations to crop production causing significant crop loss. However, understanding the physiological, biochemical and molecular responses of plants to stress and the identification of the regulatory pathways responsible for plant adaptation/tolerance to stress remains one of formidable challenges to plant science researchers around the world. Significant achievements have been recently made in understanding gene function during abiotic stress and a great number of transformed plants have been generated expressing a single or multiple genes with the aim to change metabolic pathways to obtain higher stress tolerance in plants. In contrast to pest-resistant transformed plants, where the resistance mechanism might rely on a single gene, such as the *Bt* gene (Sharma et al., 2000), the multi-loci nature for abiotic stress tolerance still renders it difficult to generate stress-tolerant plants (Bajaj *et al*., 1999; Iba, 2002; Wang *et al*., 2003). Several studies have previously shown that the introduction of a single gene into the plant genome has rarely resulted in the desirable phenotype of abiotic stress tolerance (Chen and Murata, 2002; Wang *et al*., 2003). This might be partly due that such gene is not expressed at a level required for stress protection.

At the onset of this PhD study, the the working hypothesis was to investigate whether the expression of a transgene in genetically engineered plant confer tolerance to frequently co-existing abiotic stresses, such as drought/heat, to which plants are often exposed under natural field conditions. For this, an exogenous rice cysteine proteinase inhibitor (OC-I) gene was expressed in transformed tobacco under the control of the constitutive 35S CaMV promoter. As a first new finding, this study has shown that exogenous OC-I

expression was not affected by drought, heat or a combination of both stresses. OC-I was also active *in vitro* providing evidence of maintenance of the structural integrity and function of the protein in a transformed plant under these stresses. Transformed plants were even able to accumulate active OC-I under stress exposure.

As a second new finding, this study has also shown that exogenous OC-I expression does not provide significant protection against the decline in plant performance due to drought/heat stress. Therefore, the working hypothesis that OC-I could provide stress protection in transformed tobacco could not be proved in this study. This is in contrast to other stress-tolerannce functional gene, such as compatible solutes (e.g. P5CS), HSPs and molecular chaperons (e.g. HVA1) or signal transduction and regulatory genes such as NPK1 and DREB1. These have been previously used to enhance abiotic stress tolerance in transformed plants including *Arabidopsis* and tobacco (Kasuga et al., 1999; Hong *et al*., 2000; Sivamani *et al*., 2000; Konstantinova *et al*., 2002; Shou *et al*., 2004).

As a third new finding, this study has shown that OC-I expression very likely affects gene expression. This result was found when OC-I expressing and non-expressing plants were compared and the cDNA-RDA technique was applied. One of the sequences identified to be affected by OC-I expression was the chlorophyll *a/b* binding protein gene of photosystem II. This gene was down-regulated in OC-I expressing plants in the presence and absence of heat stress. Differential expression of the chlorophyll *a/b* binding protein gene expression has been further found to be associated with lower chlorophyll content.

This might also be partially responsible for the difference found in plant performance between OC-I expressing and non-expressing plants.

As a fourth new finding, this study has provided first evidence that OCI-expression protects against protein degradation. Results of this study showed that expression and degradation of certain proteins was less affected when exogenous OC-I is expressed in a plant. OC-I possibly interacts with an endogenous proteinase(s) being responsible for protein degradation. However, this target proteinase(s) for OC-I in tobacco are currently still unknown.

As a fifth new finding, this study provided first information about such possible target proteinases. Two previously un-described papain-like cysteine proteinases could be isolated and characterized from green and senescent tobacco leaves. These proteinases might be a useful tool in future to obtain a more detailed understanding of possible OC-I cysteine proteinase interactions during plant development in a transformed plant. A study of this interaction might further help to also elucidate possible further benefits for plants expressing an exogenous proteinase inhibitor including the identification of proteins that are protected by OC-I expression from degradation.

Overall, this study has contributed to the advancement of science by providing advanced knowledge about involvement and stability of an exogenous phytocystatin in drought/heat stress, the effect of phytocystatin over-expression on gene regulation/expression and the identification of a first set of cysteine proteinases which might interact with a constitutively expressed exogenous phytocystatin.

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