University of Pretoria etd, Beyene G (2006)

CHAPTER ONE

GENERAL INTRODUCTION

University of Pretoria etd, Beyene G (2006)

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).

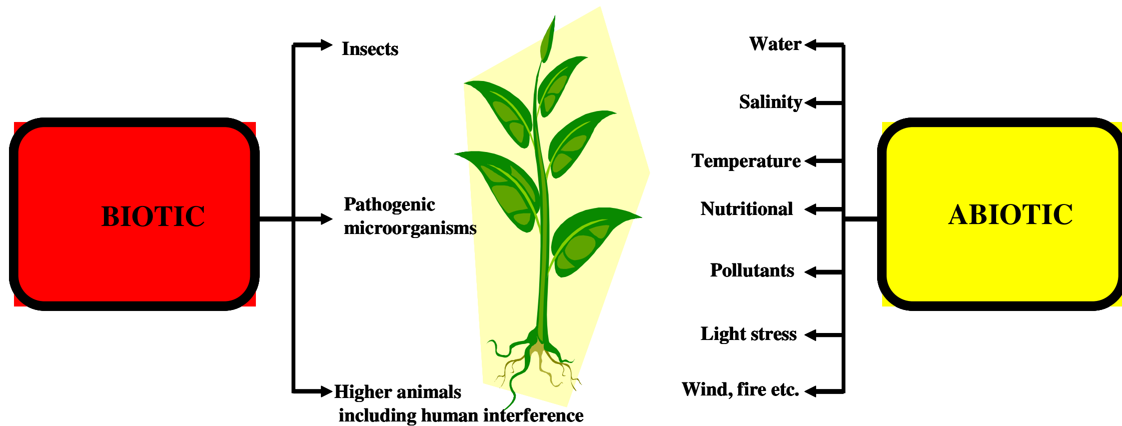


Figure 1.1 Biotic and abiotic stresses that affect plant growth and development

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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).

University of Pretoria etd, Beyene G (2006)

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).

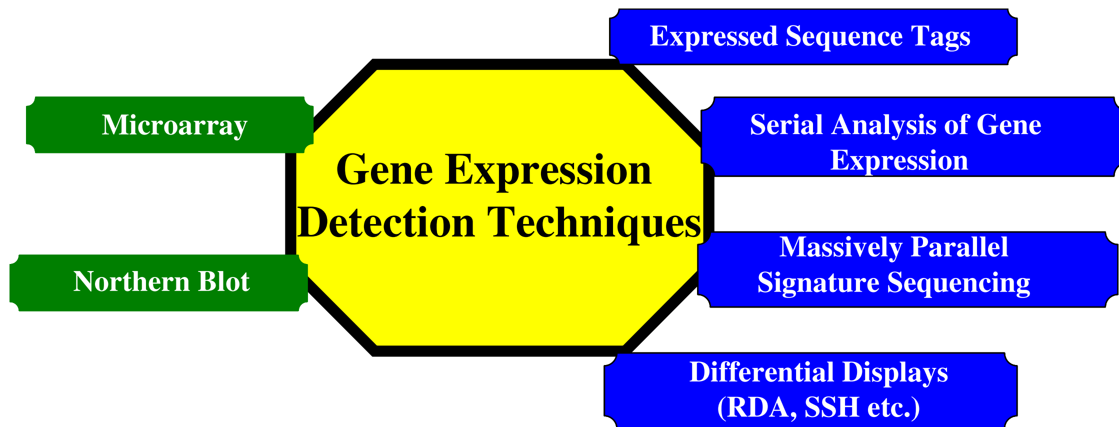


Figure 1.2 Techniques for gene expression profiling

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

University of Pretoria etd, Beyene G (2006)

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.

University of Pretoria etd, Beyene G (2006)

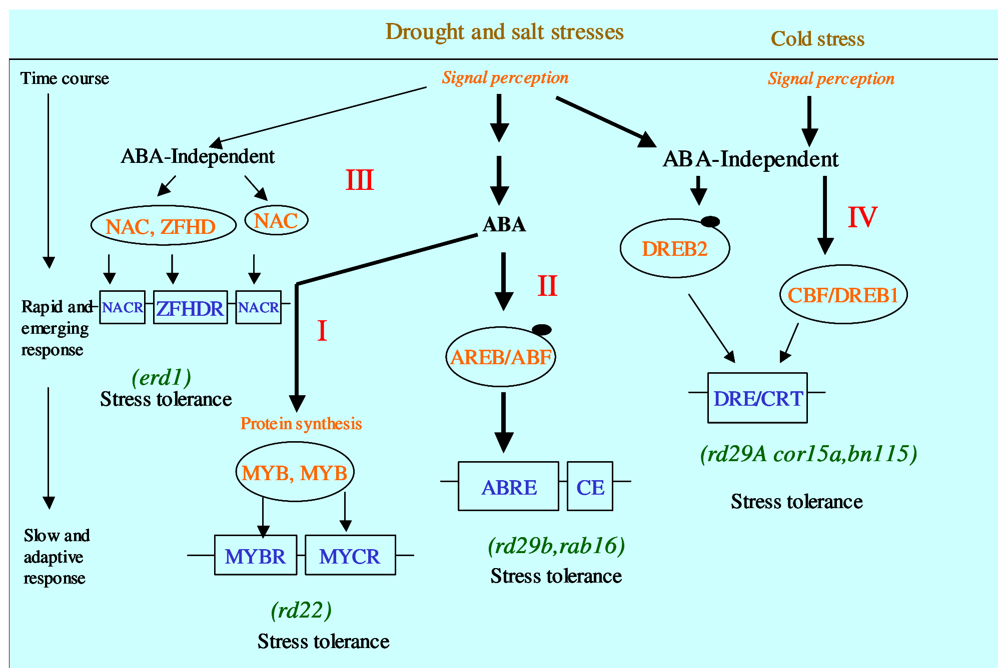


Figure 1.3 Regulatory networks of *cis*-acting elements and transcription factors involved in osmotic- and cold-stress responsive gene expression. Transcription factors controlling stress-inducible gene expression are depicted as ellipses. C*is*-acting elements involved in stress responsive transcription 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).

University of Pretoria etd, Beyene G (2006)

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.

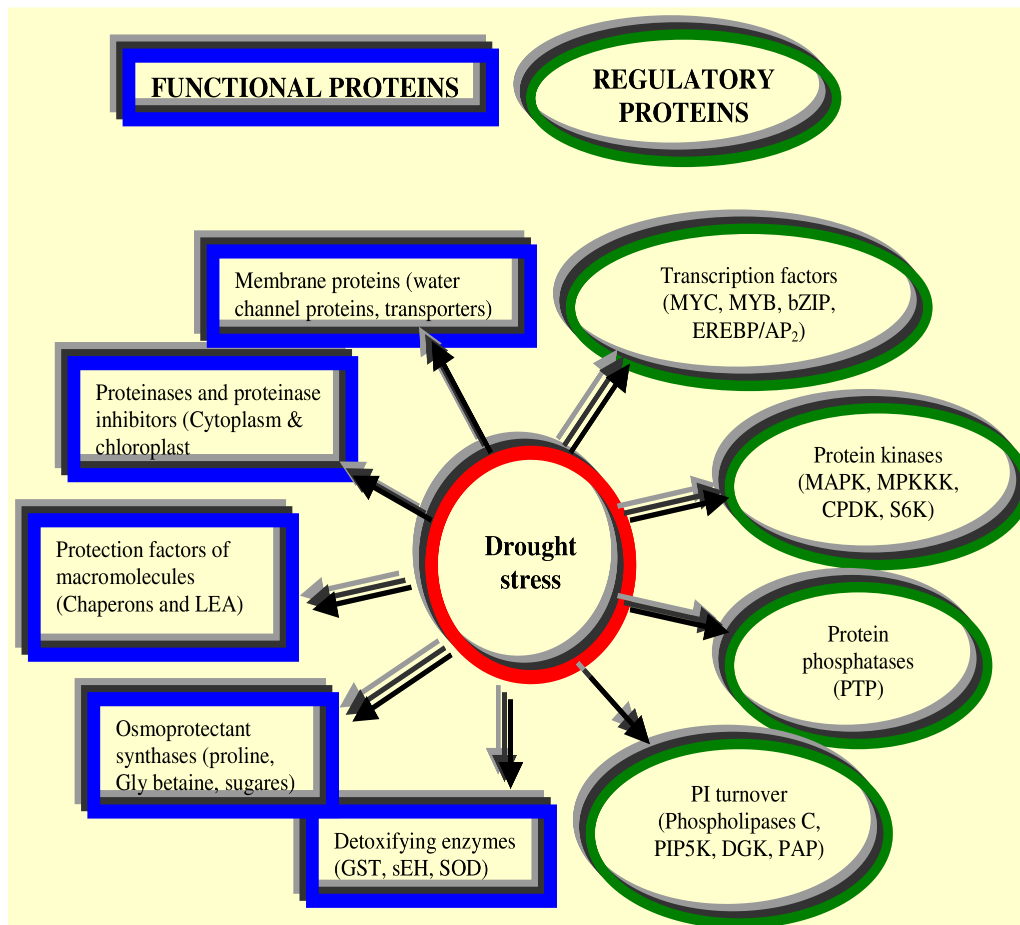


Figure 1.4 Drought stress inducible genes and their possible functions in stress tolerance and response. Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (functional proteins), and the second group contains protein factors involved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

University of Pretoria etd, Beyene G (2006)

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).

University of Pretoria etd, Beyene G (2006)

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).

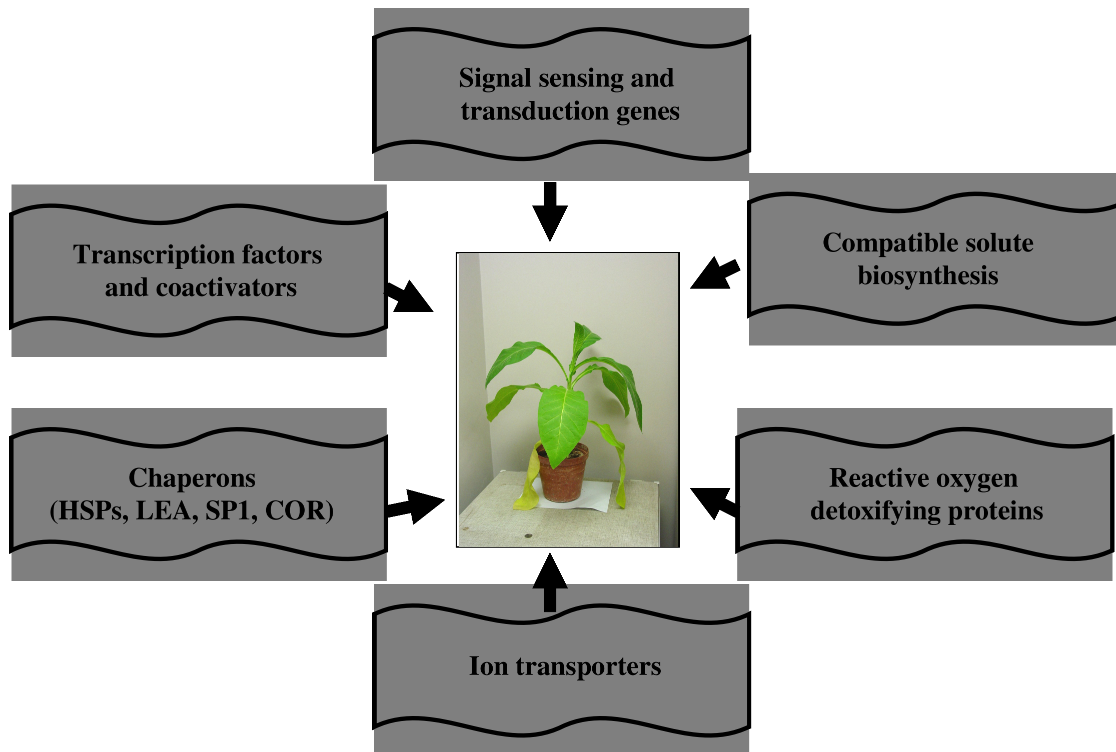


Figure 1.5 Genes that have been used to enhance abiotic stress tolerance in plants.

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).

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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.

University of Pretoria etd, Beyene G (2006)

1.4 Proteinase/proteinase inhibitor system and stress

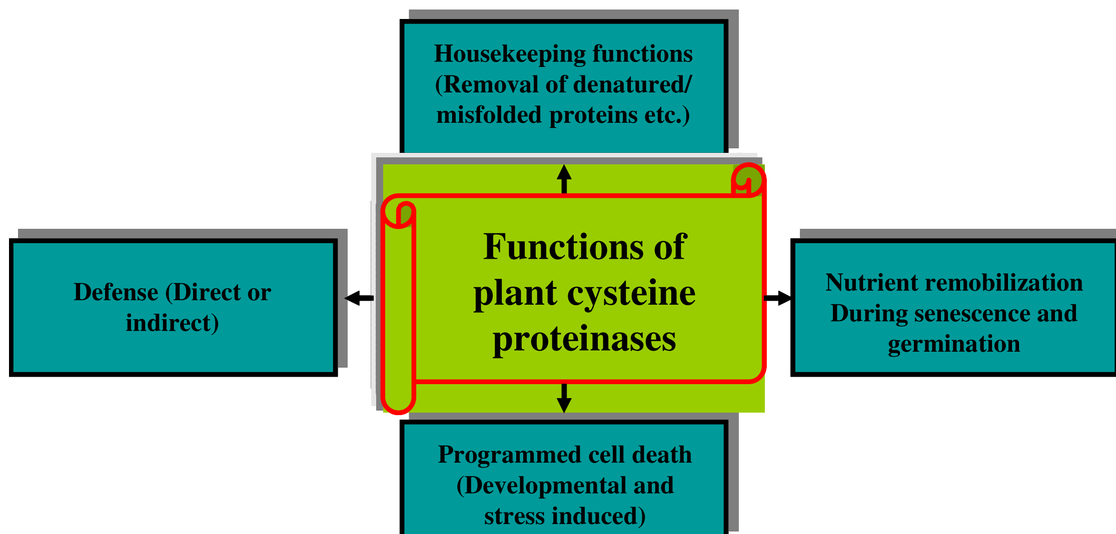
1.4.1 Plant proteinases

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).



University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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.,*

University of Pretoria etd, Beyene G (2006)

			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*

University of Pretoria etd, Beyene G (2006)

	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.

University of Pretoria etd, Beyene G (2006)

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.*

University of Pretoria etd, Beyene G (2006)

(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).

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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*.,

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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 Rotylechulus reniformis*	*Vain et al., 1998 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-

University of Pretoria etd, Beyene G (2006)

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

University of Pretoria etd, Beyene G (2006)

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).