CHAPTER 1: Introduction

1.1 Approaches for crop improvement and resistance to biotic and abiotic stress

Since the first transgenic tobacco plants expressing foreign proteins were produced (Horsch et al., 1985), genetic engineering approaches have become a routine in plant science laboratories around the world (Table 1.1). However, the introduction of a useful single well-defined and optimized gene is still a major focus for research in many crop species (Altpeter et al., 1999; Repellin et al., 2001; Langridge et al., 2006). There is also a need for the identification and evaluation of novel DNA sequences that will be useful in increasing the tolerance of plants to the often extreme environmental growth conditions experienced in Africa (Langridge et al., 2006). Factors that increase tolerance to pest species and extreme heat and drought are also critical to the future success of African agriculture. Hence, achieving enhanced resistance to biotic and abiotic stresses is one of the most important challenges to plant scientists in Africa.

Table 1.1 Examples of successful expression of transgenes in plants by genetic engineering.

Genes of plant origin are indicated in bold. (From Babu et al., 2003)

Introduced trait	**Chimeric genes**
Abiotic stress tolerance	***gpat***, ***sod***, *MtlD*
Salinity tolerance	*betA*, ***p5cs***, *halI, codA, afp, imtI*
Fungal resistance	Chitinase, ribosome inactivating protein (RIP)
Virus protection	Coat protein genes, antisense-coat protein, satellite RNA
Insect resistance	Bt toxin; *cryIA(a), cryIA(b), cryIA(c), cryIC, cryIIIA*, **protease inhibitor (*CpTi*), corn cystatin (CC), oryza cystatin I (OCI), α-AI, *gna***, chicken *avidin* gene
Herbicide tolerance	*aro A* and EPSP (glyphosate), *bar* (phosphinothricin), *bxn* (bromoxynil), *ALS* (sulfonylurea), *tfdA* (2,4-D)

The use of protease inhibitors in the improvement of plant tolerance to insects has received a lot of interest. Since the first successful increase in insect resistance was achieved by introduction of a trypsin inhibitor (Hilder et al., 1987), plant-derived protease inhibitor genes have been viewed as attractive targets in approaches geared to increasing the insect resistance of crop plants (Hilder and Boulter, 1999; Ussuf et al., 2001; Ferry et

al., 2004). Early examples include expression of a potato serine protease inhibitor applied in cowpea and rice (Duan et al., 1996), a Kunitz protease inhibitor in poplar (Colfalonieri et al., 1998) and a trypsin inhibitor in rice (Mochizuki et al., 1999).

Relatively few studies have incorporated more than one transgene. However, the inclusion of protease inhibitors together with other transgenes conferring insect tolerance can enhance the effectiveness of the latter (Sharma et al., 2004). For example, Boulter et al (1990) showed enhanced resistance to *Heliothis virescens* (tobacco budworm) by introduction of both the cowpea trypsin inhibitor and the pea lectin into tobacco plants. In addition, Bt activity can be enhanced in transgenic plants by simultaneous expression of serine protease inhibitors (MacIntosh et al., 1990). The interaction between proteases and protease inhibitors and their enzyme substrates in transgenic plants is therefore important but has been relatively poorly studied to date.

Traditional approaches designed to improve abiotic stress tolerance in crop plants have exploited the natural diversity of more-resistant varieties in breeding programs (Akbar et al., 1986; Yamauchi et al., 1993; Flowers and Yeo, 1995; Moons et al., 1995; McCouch, 2005). However, classic genetic approaches are difficult to implement, largely because of the complex multi-genic nature of the tolerance traits (Jain and Selvaraj, 1997; Nguyen et al., 1997; Khanna-Chopra and Sinha, 1998; Flowers, 2004; Vinocur and Altman, 2005). The abiotic factors that cause stress include sub- and supra-optimal temperatures, excess salt (mainly NaCl) levels, reduced water availability leading to dehydration stress, as well as the cellular oxidative stress caused by sub- and supra-optimal environmental conditions (Grover et al., 1999; Knight and Knight, 2001; Chinnusamy et al., 2004). Many of these stress factors occur simultaneously, resulting in a compound effect. For example, drought is often accompanied by high temperature stress; salt stress often results in water deficits, while low temperature stress is frequently associated with drought stress. In addition, oxidative stress results from exposure to a wide variety of stresses like excess light, excess or shortage of water, and extreme temperatures.

Survival of stressful environments requires extensive acclimation of most if not all of the major metabolic processes including photosynthesis, nitrogen fixation, nitrogen metabolism and respiration (Grime, 1989; Nguyen et al., 1997; Pareek et al., 1997; Khanna-Chopra and Sinha, 1998; Knight and Knight, 2001; Chinnusamy et al., 2004;

Maestre et al., 2005). Since plant growth and development respond to environmental cues, it is perhaps not surprising that exposure to stress causes many physiological and morphological adjustments, and these can effect plant productivity at every developmental stage (Khanna-Chopra and Sinha, 1998; Maestre et al., 2005; Mittler 2006).

Transgenic approaches to the improvement of plant stress tolerance require identification of genes of interest that will not only protect plants but enable them to maintain vigour under abiotic stress conditions. Global expression profiling has identified large numbers of genes involved in plant stress responses (Cheong et al., 2002; Kreps et al., 2002; Jiang and Zhang, 2003). Comparisons of multiple stresses at different time points has allowed the identification of transcript changes that underpin stress-specific and "common" or "shared" responses (Fig. 1.1; Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001; Kreps et al., 2002; Fujita et al., 2006).

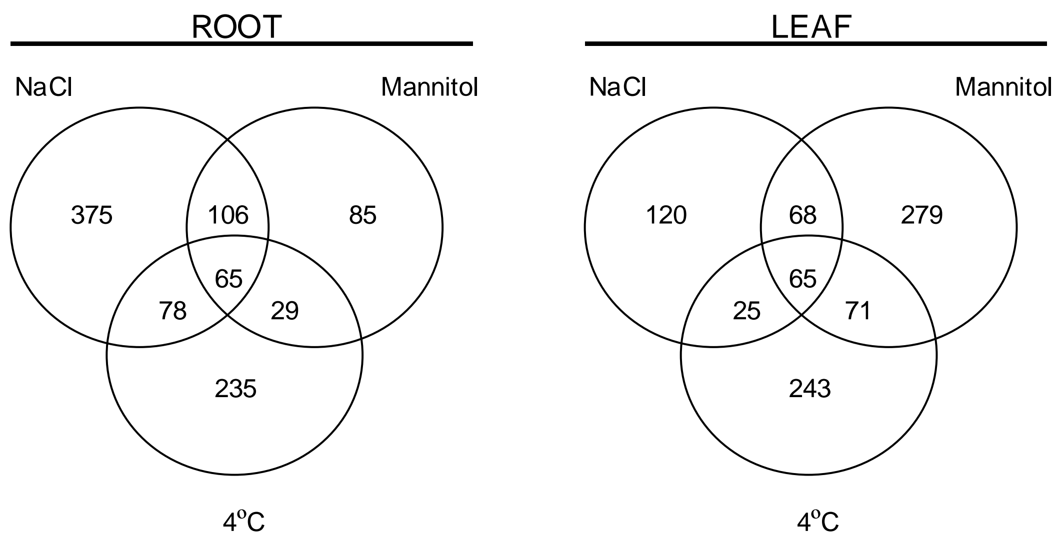

Figure 1.1 Venn diagrams showing the distribution of stimulus-specific and shared stress responses (> 2-fold) in *Arabidopsis thaliana*. These changes were observed after 3 hours of exposure to the stress applied, either NaCl, mannitol, or cold stress (4°C). (From Kreps et al., 2002).

A rather large number of individual genes are effective in improving abiotic stress tolerance using transgenic approaches. Some of these encode enzymes involved in metabolic pathways that confer stress tolerance, such as glycerol 3-phosphate acyltransferase (Murata et al., 1992; Grover et al., 2000; Iba, 2002) mannitol-1 phosphate dehydrogenase (Tarczynski et al., 1993; Chen and Murata, 2002) and superoxide dismutase (Aono et al., 1995; McKersie et al., 1999; Van Breusegem et al., 1999;

McKersie et al., 2000). A number of studies have involved modified expression of osmolytes (Yancey et al., 1982; Morgan, 1984; Vernon et al., 1993; Kavi Kishor et al., 1995; Hayashi et al., 1997; Romero et al., 1997; Shen et al., 1997). Many of the studies that have focused on the enhancement of the antioxidant defences have met with mixed success (Foyer and Noctor, 2003). The mechanisms by which some selected transgenes confer tolerance to abiotic stresses are more obscure. For example, transgenic plants with decreased poly (ADP-ribose) polymerase (PARP) levels show broad-spectrum stress-resistant phenotypes. This increase in stress tolerance was initially attributed to an improved maintenance of cellular energy homeostasis due to reduced NAD(+) consumption (De Block et al., 2005). However, it has recently been shown that PARP2-deficient *Arabidopsis* plants also have higher leaf abscisic acid contents and this could also explain the observed induction of a wide set of defence-related genes in the transgenic plants (Vanderauwera et al., 2007).

The C promoter-binding factor 1 (CBF1) transcription factor, which controls the cold-regulated *cor* genes has been used to increase the freezing tolerance of plants and dehydration response element B1A (DREB1A), a drought-responsive element binding protein was found to enhance both freezing and dehydration tolerance (Liu et al; 1998; Gong et al., 2002; Pino et al., 2007). However, transgenic plants over expressing DREB1A also showed a dwarfed phenotype (Pellegrineschi et al., 2004). Genes that play a role in signal transduction pathways involved in regulating stress responses have also been used in transgenic approaches to improve stress resistance (Liu and Zhu, 1998). More recent approaches have focused on other signal transducing components, particularly the mitogen-activated protein (MAP) kinase pathways (Lee and Ellis, 2007).

While transgene technologies have greatly improved over the intervening years and many genes that are important in plant stress responses have been identified, there remains a large gap in our current ability to translate information gained in the laboratory into enhanced vigour under stressful environmental conditions. In part this is due to the absence of a comprehensive characterisation of gene function in crop plants. Much work has focused on the analysis of stress responses in model species. Moreover the functions of stress markers and stress-related genes have been studied largely in model plants, such as tobacco and *Arabidopsis* (Vinocur and Altman, 2005). While such studies are vital, it is also important to identify genes that are involved in the stress responses of crop plants and

to study their function in the context of overall yield in the field as well as under controlled environment conditions.

1.2 The use of protease inhibitors for crop improvement

The application of protease inhibitors in plant protection was investigated as early as 1947, when Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Mickel and Standish, 1947). Subsequently, it was shown that trypsin inhibitors present in soybean are toxic to the larvae of the flour beetle, *Tribolium confusum* (Lipke et al., 1954). Other investigations revealed that plant protease inhibitors can inhibit insect gut proteases; both *in vitro* (Pannetier et al., 1997; Koiwa et al., 1998) and *in vivo* using artificial diet bioassays (Urwin et al., 1997; Vain et al., 1998; Foissac et al., 2000; De Leo et al., 2001; Ferry et al., 2004). There has been a large amount of interest in the use of plant protease inhibitors in transgenic approaches for the improvement of crop resistance to both biotic and abiotic stresses. In addition to improved stress tolerance, the application of protease inhibitors has three additional advantages. Firstly, transgenic crops expressing plant-derived protease inhibitors are useful in integrated pest management (IPM) systems that aim to minimise pest damage to crops without the application of harmful pesticides (Boulter, 1993). Secondly, protease inhibitors improve the nutritional quality of food as many of them are rich in cysteine and lysine (Ryan, 1989). Thirdly, protease inhibitors can protect heterologous proteins when both are expressed together in transgenic potato (Rivard et al., 2006). Hence, protease inhibitors have great potential in large-scale production systems for recombinant proteins in plants. This transgenic approach is a viable, safe, and useful option, especially when considering the expression of biologically active mammalian proteins that require essential post-translational modifications. It is widely assumed that ectopic protease inhibitor expression has little effect on plant growth and development but this has not been studied intensively in most cases. Furthermore, it is of interest to determine whether ectopically expressed protease inhibitors affect the action of key endogenous proteases involved in essential proteolysis functional during development, stress response, and senescence. Of the wide variety of protease inhibitors that occur in plants, only one family, the cystatins, has been studied in great detail.

1.2.1 Cysteine protease inhibitors (Cystatins)

The cystatin superfamily is composed of cysteine protease inhibitors that bind tightly and reversibly to the papain-like cysteine proteases present throughout the animal and plant kingdoms (Nicklin and Barret, 1984; Margis et al., 1998). All members of the superfamily exhibit similarities in their amino acid sequences and functions (Barret et al., 1986). Three distinct families were originally classified: the cystatin family consisting of groups of small proteins with two disulfide bonds; the stefin family of small proteins (~12 kDa) lacking disulfide bonds; and the kininogen family of large glycoproteins (60-120 kDa) containing three repeats similar to those found the cystatin family (Barret et al., 1986). The plant cystatins have structural peculiarities, genomic arrangements and intrinsic diversity compared to animal cystatins. This led to the creation of a new family, the phytocystatins (Kondo et al., 1991; Margis et al., 1998).

Cystatins are part of an innate plant bio-defence system against insect attack (Michaud et al., 1993b; Matsumoto et al., 1995; Michaud et al., 1995; Edmonds et al., 1996; Kuroda et al., 1996 Matsumoto et al., 1997; Matsumoto et al., 1998). This has lead to research using transgenic approaches involving cystatins in insect control (Benchekroun et al., 1995; Urwin et al., 1995; Irie et al., 1996; Arai et al., 2000). Cystatin expression can have other beneficial effects, for example, expression of the rice phytocystatin Oryzacystatin-I (OC-I; Fig. 1.2) in transformed tobacco improved recovery of photosynthesis following cold stress (Van der Vyver et al., 2003).

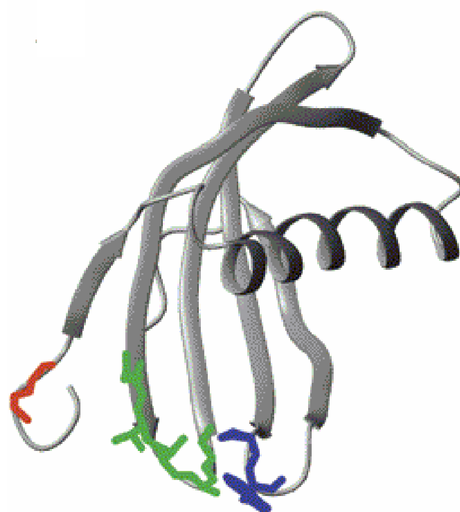

Figure 1.2 Structure of the rice phytocystatin OC-I (Nagata et al., 2000).

A number of investigations concerning cystatin expression have focussed on seeds. (Kondo et al., 1990; Abe et al., 1995; Kuroda et al., 2001). Less information is available on other tissues. A sorghum cystatin was reported in vegetative tissues (Li et al., 1996). The barley cystatin, Hv-CPI, was detected in embryos, developing endosperm, leaves and roots. Hv-CPI expression increased in vegetative tissues in response to stress, particularly anaerobiosis, dark and cold shock (Gaddour et al., 2001). Many phytocystatins are induced upon exposure to abiotic stress (Pernas et al., 2000) and biotic stresses (Leple et al., 1995). Cysteine proteases are key enzymes in animal apoptosis and they may have similar roles in plant programmed cell death (PCD) as ectopic expression of cystatins was found to block H_2O_2-induced cysteine protease activity during PCD (Solomon et al., 1999). In such situations plant cystatins may protect against invasion by viruses, bacteria, and insects as they can inhibit cysteine proteases from a wide range of organisms. The relationships between the expression of cysteine proteases and that of cystatins is poorly characterised. The cathepsin B-like cysteine protease (gene *CatB*) was found to show similar patterns of expression to the *Icy* cystatin in barley vegetative tissues (Martínez et al., 2002). The *Icy* encoded protein is a potential inhibitor of several cysteine proteases including the CatB protease. Leaf *CatB* and *Icy* mRNAs show similar circadian expression patterns of regulation and they are similarly induced by chilling. However, these genes showed different expression patterns in pre and post-germinating embryos and they had different hormonal responses in the aleurone layers.

While protease inhibitors have been used ectopically to improve biotic and abiotic stress resistance, little research has focused on the effect of this ectopically-expressed inhibitor on possible endogenous protease targets. Interaction between an exogenous protease inhibitor and an endogenous protease will potentially affect the mechanism of proteolysis which is very important in the process of metabolic change due to development or stress response. It is important to understand the function of protein degradation as well as the role of important proteases in the plant, in order to understand where exogenous protease inhibitors might affect plant metabolism.

1.3 Protein degradation and proteases

Proteolytic enzymes play a crucial role in plant defence and acclimation to changing metabolic demands and environmental cues, as well as in the orchestration of plant development from regulation of cyclin lifetime in the cell cycle to the programming of

senescence. These important enzymes are responsible for the post-translational modification of the cellular protein network by limited proteolysis at highly specific sites (Vierstra, 1996, Beers et al., 2000). Limited proteolysis participates in the control of enzyme activity as well as the functioning of regulatory proteins and peptides (Callis and Vierstra, 2000). It is used by the cell to control the production, assembly and sub-cellular targeting of mature enzyme forms.

Proteolytic enzymes play a vital role in protein turnover. They are essential components of the cellular protein homeostasis and repair system, removing damaged, mis-folded, or harmful proteins as well as limiting the lifetime of proteins such as the DELLA proteins that control plant growth and development (Vierstra, 1996; Frugis and Chua, 2002). The selective breakdown of regulatory proteins by the ubiquitin-proteasome pathway (Fig. 1.3) controls key aspects of plant growth, development, and defence (Hochstrasser, 1995; Hellman and Estell, 2002; Vierstra, 2003; Dreher and Callis, 2007).

1.3.1 The ubiquitin/proteasome system

Proteins that are targeted for degradation by ubiquitin/proteasome system are first modified by the addition of ubiquitin. The polyubiquitin chains are covalently linked to a lysine residue in the target protein (Pickart, 2001) in a sequential cascade of enzymatic reactions (Fig. 1.3). A uibiquitin-activating enzyme (E1) first binds to the G76 residue of ubiquitin (1). This enzyme transfers the ubiquitin moiety to a ubiquitin-conjugating enzyme (E2) (2), which carries the activated uibiquitin to a ubiquitin ligase (E3) (3), which facilitates the transfer of the ubiquitin to a lysine residue in the target protein (4). This process is repeated for the formation of a polyubiquitin chain on the target protein (5). In *Arabidopsis* two genes encode ubiquitin-activating enzymes, at least 45 genes encode ubiquitin-conjugating enzymes, and almost 1200 genes encode ubiquitin ligases (Vierstra, 2003). The type of chain synthesised determines the fate of the protein that has polyubiquitin chains. Chains formed at the K48 residue of ubiquitin are destined for degradation by the 26S proteasome. The proteasome degrades the target protein and the ubiquitin monomers are reclaimed by the action of de-ubiquitinylating enzymes (Vierstra, 2003).

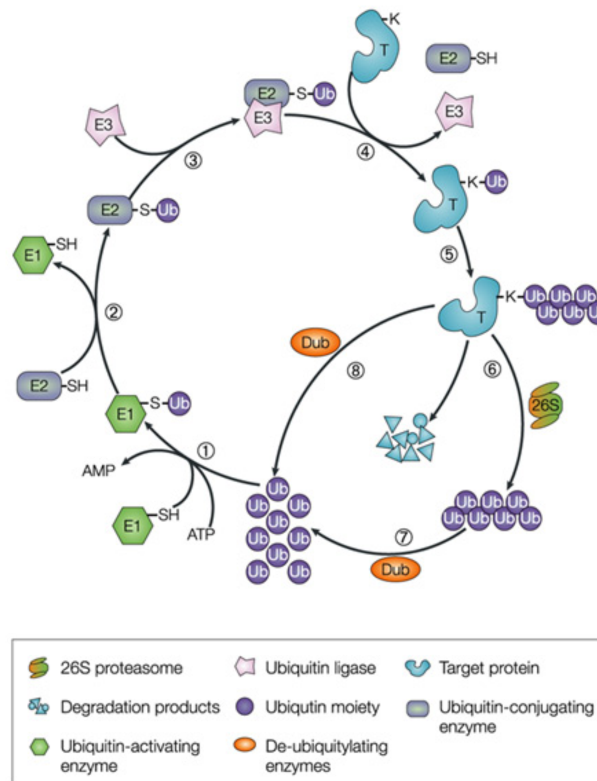

Figure 1.3 The Ubiquitin/Proteasome system. (Sullivan et al., 2003). Process described in text.

The 26S proteasome consists of the 20S core protease and the 19S regulatory particle (Fig. 1.4; Voges et al., 1999). The core protease is a broad-spectrum ATP- and ubiquitin - independent peptidase created by the assembly of four, stacked heptameric rings of related α and β subunits in a $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ configuration. The protease-active sites within the β1, β2 and β5 polypeptides are sequestered in a central chamber. Access to this chamber is restricted by a narrow gated channel created by the α-subunit rings that allows only unfolded proteins to enter (Glickman, 2000). Each end of the central protease is capped by a regulatory particle. The regulatory particle confers both ATP-dependence and substrate specificity to the holoenzyme (Voges, 1999; Glickman, 2000). The regulatory protein is able to identify appropriate substrates for breakdown, releasing the attached ubiquitins and opening the α-subunit ring gate. This directs the entry of unfolded proteins into the central protease lumen for degradation.

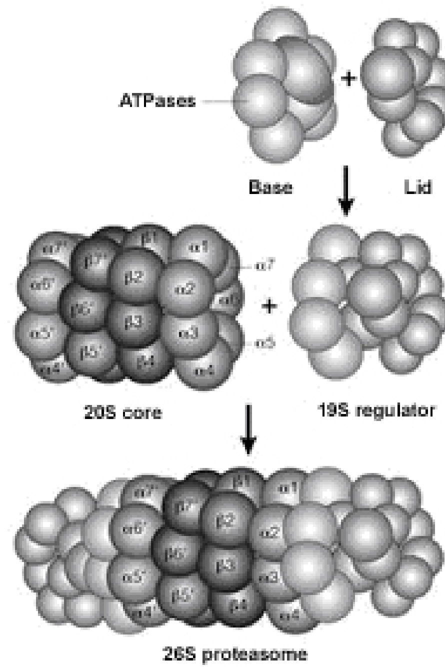

Figure 1.4 The 20S core protease and 19S regulatory protein that constitute the 26S proteasome complex (Kloetzel, 2001).

1.3.2 Proteases

Proteases are responsible for degradation of proteins that are not uibiquitinated. They are divided into four classes according to catalytic mechanism: serine, cysteine, aspartic, and metalloproteases (Fig. 1.5; Fan and Wu, 2005). A further class has been suggested for proteases of unidentified catalytic mechanism. Since the research described in this thesis largely concerns inhibitors of only serine and cysteine proteases, these categories alone are discussed in detail below.

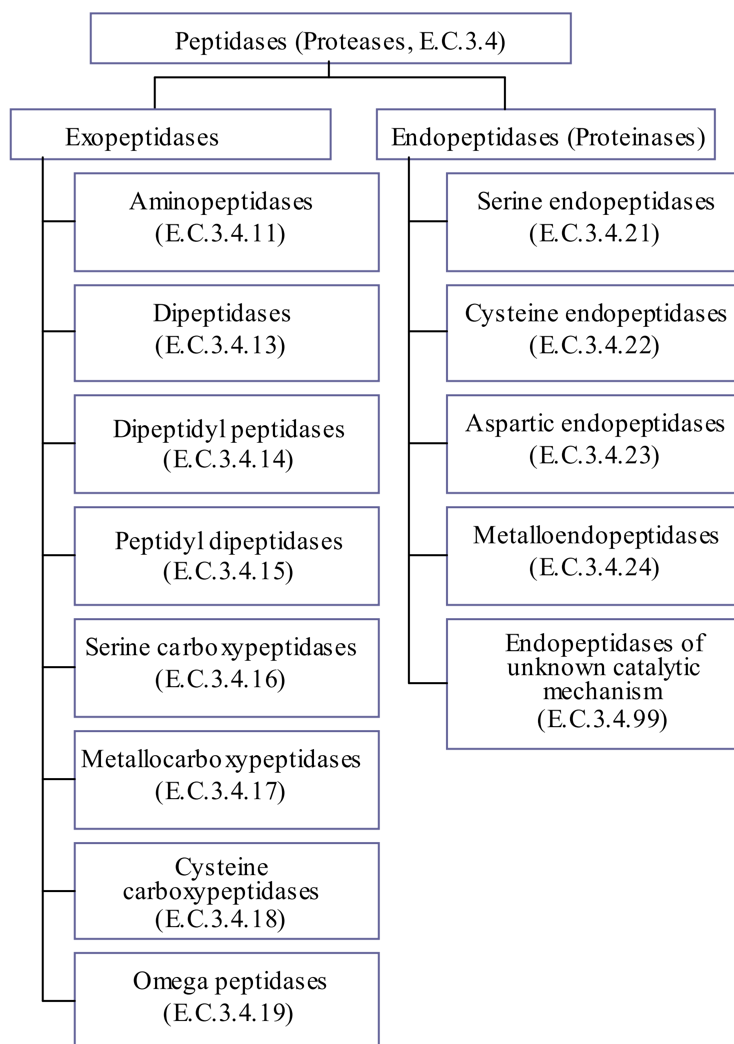

Figure 1.5 The main classes of proteases (peptidases) according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 1992). NC-IUBMB recommended the term peptidase as the general term for all enzymes that hydrolyse peptide bonds. This is subdivided into exopeptidases cleaving one or a few amino acids from the N- or C-terminus, and endopeptidases cleaving internal peptide bonds of polypeptides. The classification of exopeptidases is based on their actions on substrates while the endopeptidases are divided by their active sites. Proteases are divided into four groups: the serine proteases, the cysteine proteases, the aspartic proteases, and the metalloproteases. (From Fan and Wu, 2005)

Cysteine proteases, senescence, and programmed cell death (PCD)

Cysteine proteases have roles in the resistance of plants to attack by pathogens (Krüger et al., 2002) and insects (Pechan et al., 2000; Konno et al., 2004) and they also have well-characterised functions in senescence and PCD (Solomon et al., 1999; Wagstaff et al., 2002; Belenghi et al., 2003; Okamoto et al., 2003). Their roles in nitrogen remobilisation

have been well characterised, particularly in regard to the degradation of the storage proteins (Kato and Minamikawa, 1996; Tooyoka et al., 2000; Gruis et al., 2002).

Leaf senescence is a complex and highly coordinated developmental process that precedes plant death, and in which proteolysis plays a major part (Smart, 1994; Nooden et al, 1997; Gepstein et al, 2003). Senescence-associated genes (SAGs) are considered to be specifically up-regulated during senescence (Bleecker and Patterson, 1997; Gepstein et al., 2003). These include genes for proteases (Dangl et al., 2000; Lohmann et al., 1994; Thompson et al., 1998, 2000), including those of the cysteine protease family (Chen et al, 2002). It is generally believed that proteases active during senescence function in the remobilization of nitrogen to sink tissue. Two cysteine proteases, SAG2 and SAG12 have proved to be particularly important senescence markers. SAG2 shows senescence-enhanced expression while SAG12 shows senescence-specific expression (Hensel et al. 1993, Lohman et al. 1994; Grbić, 2003). SAG2 shows sequence similarity to cathepsin H and SAG12 to cathepsin L (Hensel et al. 1993, Lohman et al. 1994). The low level of SAG2 expression in young leaves indicates that this protease functions in protein turnover throughout the life of the leaf. In contrast, the specific induction of SAG12 at the onset of leaf senescence indicates that it has a more specialized role in protein breakdown during senescence. Cathepsin cysteine proteases are active at acidic pH, and are therefore assumed to be localised in lysosomes or vacuoles (McGrath 1999; Turk et al., 2001).

The process of PCD in plant cells has been compared to apoptosis in animal cells (Jones and Dangl, 1996; Elbaz et al., 2002; Van Doorn and Woltering, 2005). In animal cells the major regulators of apoptotic cell death are the caspases, a family of cysteine proteases with specificity for asparagine (Asp; Shi, 2002). When an apoptotic signal is perceived, inactive caspases are processed to their active states by oligomerization and subsequent conformational changes (Fuentes-Prior and Salvesen, 2004). This irreversible activation triggers a proteolytic cascade that activates the enzymes involved in apoptosis. While caspase inhibitors can block PCD in plants (Del Pozo and Lam, 1998; Watanabe and Lam, 2004) they have not evolved the apoptotic sequence of activation of canonical caspases or the pro-and anti-apoptotic functions of Bcl-2 family proteins. However, caspase homologs are present in plants based on sequence similarity. These have been designated metacaspases (Uren et al., 2000) but they have no known function. For example, constitutive overexpression or disruption of metacaspase genes in *Arabidopsis* does not

result in an obvious phenotype (Vercammen et al., 2006; Belenghi et al., 2007). However, micro-array analysis on the expression of over 20,000 *Arabidopsis* genes (Zimmermann et al., 2004) showed that metacaspases are induced in response to biotic and abiotic stresses (Sanmartín et al., 2005). Certain metacaspase genes are also strongly induced in senescing flowers (Sanmartín et al., 2005). Moreover, the activity of the *Arabidopsis* metacaspase, AtMC9, is inhibited by AtSerpin1, a serine protease inhibitor that inhibits AtMC9 strongly through binding and cleavage of its reactive center loop (Vercammen et al., 2006).

In contrast to animals, dead cells in plants are never removed by phagocytosis. PCD in plants occurs by autophagasitic processes where the proteins in the dying cell are essentially removed by enzymes in growing lytic vacuoles. Most animal caspases are located in the cytosol where the proteolytic cascade is localised (Nakagawa and Yuan, 2000). However, co-operating pathways involving the vacuoles, chloroplasts, mitochondria and nucleus contribute to plant PCD, together with a repertoire of cell death proteases (Sanmartín et al., 2005). Of these, one group of proteases namely the vacuolar processing enzymes (VPEs) has been suggested to have caspase-like functions in plants (Woltering et al., 2002) particularly in PCD (Hoeberichts et al., 2003; Rojo et al., 2004). VPEs are related in sequence and in tertiary structure to animal caspases (Aravind and Koonin, 2002). VPEs also play a role in the maturation of several seed proteins (Hara-Nishimura et al., 1991, 1993), including a novel membrane protein (Inoue et al., 1995), and vacuolar proteins in leaves (Hara-Nishimura, 1998). VPEs have been identified in seeds and in vegetative tissues (Kinoshita et al., 1995). In vegetative tissues VPEs are localised in the lytic vacuoles (Kinoshita et al., 1999) while seed VPEs are localised in the protein storage vacuoles (Hiraiwa et al., 1993). VPE activities are greatly increased in the lytic vacuoles during senescence and under various stress conditions (Kinoshita et al., 1999). Moreover, VPEs are accumulated in vesicles derived from the endoplasmic reticulum (ER) in the epidermal cells in young seedlings (Hayashi et al., 2001). When seedlings are exposed to stress, these vesicles fuse with the vacuoles and perhaps other organelles and thus assist in stress-induced protein turnover and PCD.

Serine proteases

Serine proteases (carboxypeptidases) function in a wide range of plant defence processes such as PCD (Domínguez and Cejudo, 1998; Domínguez et al., 2002), and response to wounding where they are triggered by brassinosteroid signalling (Li et al., 2001). They are

also important in seed development (Cercos et al., 2003). Like the cysteine proteases, the serine proteases are involved in nitrogen remobilisation and have important functions in processes such as the remobilization of nitrogen reserves during seed germination (Antão and Malcata, 2005). The diversity of serine protease functions has been attributed to differences in substrate specificity (Walker-Simmons and Ryan 1980; Dal Degan et al., 1994; Moura et al., 2001; Granat et al., 2003).

The subtilase class of serine proteases are particularly important during microsporogenessis, where a role in signal peptide production has been suggested (Kobayashi et al., 1994). Relatively little is known about the role of the subtilisin-like serine proteases but they have been shown to function in epidermal structure and patterning. For example, *ALE1* plays a role in epidermal surface formation (Tanaka et al., 2001) and *SDD1* regulates stomatal density and distribution (Berger and Altmann, 2000). Subitilases are also produced in response to PCD triggers and have caspase-like cleavage capabilities.

Proteolysis is an essential element of metabolic change in plants whether it's initiated by development or is triggered by stimulus from the environment. One of the processes that are affected by both development and stress is the process of photosynthesis.

1.4 Photosynthesis as a target for proteolytically-mediated metabolic change

Since photosynthesis drives plant biomass production, knowledge of the responses of this process to different environmental conditions, will be vital in ensuring the success of future crop improvement strategies. As plants grow and respond to external stimuli, the photosynthetic machinery adjusts according to changing demands on photosynthate and energy. The proteins involved in photosynthesis may be controlled on post-translational level by selective proteolytic degradation. It is important to understand the different types of photosynthesis in order to determine how proteins involved in this process will be regulated.

1.4.1 C_3 and C_4 Photosynthesis

Photosynthesis is the process through which plants convert light energy into chemical energy and fix atmospheric CO_2 into organic carbon compounds (Fig. 1.6; Benson and Calvin, 1950; Edwards and Walker, 1983; Furbank and Taylor, 1995). The C_3 pathway

(also called the Calvin cycle, the Calvin-Benson cycle, or the photosynthetic carbon reduction [PCR] cycle) is the only mechanism through which plants fix CO_2 into sugar-phosphates. The C_3 cycle takes its name from the first product of the pathway, which is a three carbon compound, 3-phosphoglycerate (PGA). Two molecules of PGA are produced when CO_2 is introduced into the 5-carbon compound ribulose-1, 5-bisphosphate (RuBP), a reaction that is catalysed by ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) (Lorimer, 1981). Phosphoglycerate is then converted to two types of triose phosphate (TP) glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate by phosphorylation and reduction steps that use ATP and NADPH. For every 6 TP molecules flowing through the PCR cycle, one is produced as net product (Benson and Calvin, 1950). This can either be kept in the chloroplast where it could be used for starch synthesis, or it can be transported to the cytosol where it is used in the synthesis of sucrose or to provide carbon skeletons for the production of a wide range of other molecules including amino acids. Sucrose is then translocated from the green tissues throughout the plant for energy and biomass production.

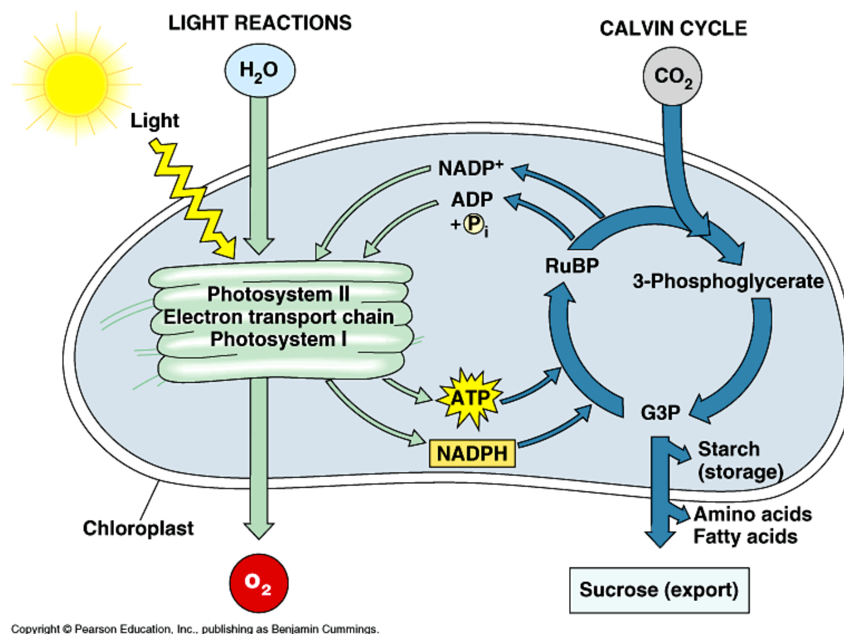

Figure 1.6 A schematic representation of photosynthesis. Light energy absorbed by photosystems I and II in the thylakoid membranes is used to produce ATP and NADPH, which drive the CO_2 assimilation (PCR) pathway in the chloroplast stroma. The PCR pathway liberates ADP and NADP which then return to the thylakoid membranes as the substrates for the light driven electron transport pathways.

RuBisCO also catalyses a second reaction in addition to CO_2 fixation (Lorimer, 1981). Molecular O_2 can replace CO_2 at the enzyme active site and the resultant fixation of O_2 into RuBP initiates a process known as photorespiration. When RuBisCO fixes O_2 it produces one molecule of 3-phosphoglycerate and one molecule of 3-phosphoglycolate rather than two molecules of 3-phosphoglycerate. The present atmospheric CO_2 concentration allows the photorespiratory pathway to operate at high rates in plants that only have the PCR cycle (C_3 plants) as O_2 efficiently competes with CO_2, particularly at higher temperatures. At current atmospheric CO_2 levels, one molecule O_2 is fixed by RuBisCO for every three molecules of CO_2 that are fixed in C_3 plants. Since 3-phosphoglycolate cannot be used in the PCR cycle, it must be recycled to phosphoglycerate through the photorespiratory pathway, which incurs additional energy costs and results in the loss of both CO_2 and nitrogen.

Certain plant species such as maize and sorghum have evolved systems that diminish the flow of carbon through the photorespiratory pathway (Edwards and Walker, 1984; Sage, 2004). In these species (called C_4) a second process has evolved to assist the C_3 pathway in CO_2 assimilation (Fig. 1.7) such that the fixation of CO_2 is a two-step process. Atmospheric CO_2 is first fixed in the cytosol of the mesophyll (M) cells by phosphoenolpyruvate (PEP) carboxylase to form a C_4 metabolite, oxaloacetate. Oxaloacetate is then converted to malate or aspartate, which diffuse into the inner ring of bundle sheath (BS) cells where they are decarboxylated in the chloroplasts. The CO_2 produced by decarboxylation is then refixed by RuBisCO. Hence, two types of photosynthetic cell in the M and BS tissues co-operate in CO_2 fixation in C_4 plants. The leaves of C_4 plants such as maize often show extensive vascularization, with a ring of bundle sheath cells surrounding each vein and an outer ring of M cells surrounding the BS. The development of this “Kranz anatomy” and the cell-specific compartmentalization of C_4 enzymes are important features of C_4 photosynthesis (Hatch, 1992, and references therein).

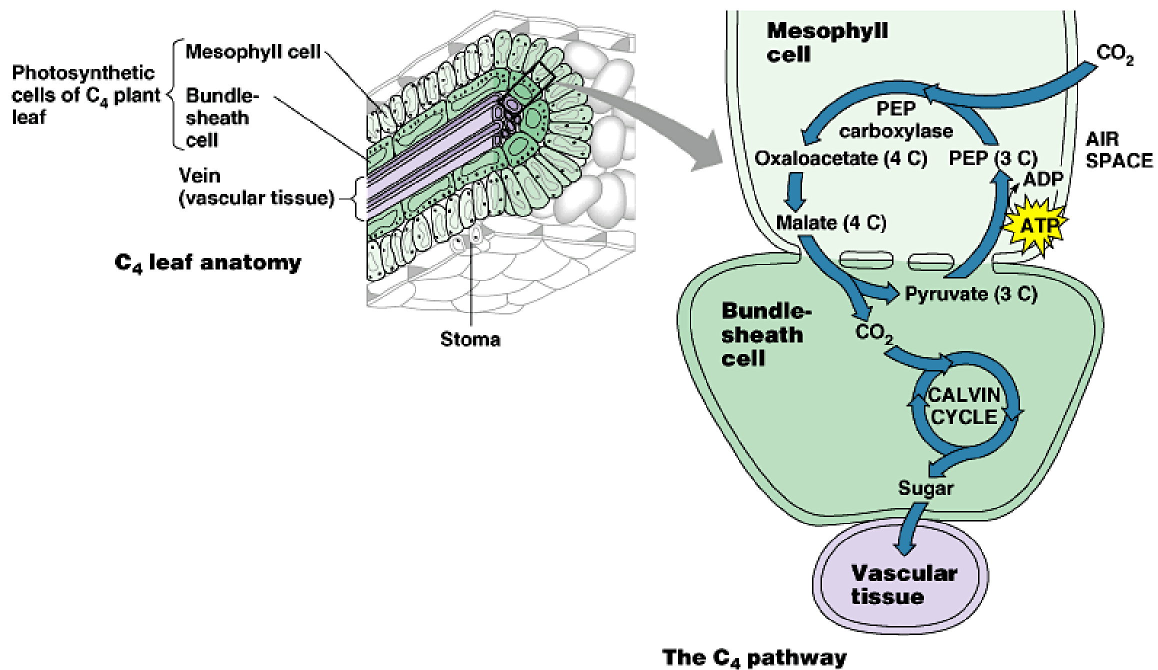

Figure 1.7 The C_4 pathway of photosynthesis. In C_4 plants the mesophyll and bundle sheath cells co-operate in the CO_2-fixing process. In this pathway, the four carbon (4C) acids that are produced by phospho*enol*pyruvate (PEP) carboxylase in the mesophyll are transported to the bundle sheath where CO_2 is liberated and fixed in to 3-phosphoglycerate by the enzymes of the Calvin (PCR) cycle.

The BS cells are separated from the M cells and from the air in the intercellular spaces by a lamella that is highly resistant to the diffusion of CO_2 (Hatch, 1992). Thus, the M-located C_4 cycle acts as an ATP-dependent CO_2 pump that increases the concentration of CO_2 in the BS to approximately 10 times atmospheric concentrations. This high CO_2 level suppresses the RuBisCO oxygenase activity which leads to higher rates of photosynthesis in C_4 plants, particularly at high light intensities and high temperatures due to the increased efficiency of the PCR cycle (Hatch, 1992).

1.4.2 Response of photosynthesis to abiotic stress

Plants need to respond quickly to changes in the environment causing changes in the demand for photosynthesis. This adaptation to environmental change often includes selective and rapid degradation of key proteins. For example, when the amount of incident light is above that which the plant can use for the process of photosynthesis, excess light damages the photosynthetic system causing photoinhibition. Photoinhibition of photo system II requires rapid acclimation with a concomitant degradation of proteins. Photoinactivation of PSII electron transcport is followed by oxidative damage to the D1

protein. The D1 protein is one of the heterodimeric polypeptides of the PSII reaction center complex. Damage to the D1 protein exposes it to an intrinsic protease (Virgin et al., 1991; De Las Rivas et al., 1992) for protein turnover. A proteolytic activity that is involved in the degradation of the major light-harvesting chlorophyll a/b-binding protein of photosystem II (LHCII) has also been identified. Degradation of this protein occurs when the antenna size of photosystem II is reduced upon acclimation of plants from low to high light intensities (Yang et al, 1998). The protease(s) involved in degradation of the major light-harvesting chlorophyll a/b-binding protein is of the serine or cysteine type and is associated with the outer membrane surface of the stroma-exposed thylakoid regions.

1.4.3 Effects of CO_2 enrichment on photosynthesis, RuBisCO, and protein turnover

The effect of increased CO_2 content in the atmosphere has recently been a matter of great interest (Stitt, 1991; Sage, 1994; Drake et al., 1997; Ainsworth et al., 2002; Nowak et al., 2004; Ainsworth and Long, 2005; Matros et al., 2006). In some important crop species a highly beneficial effect was observed. For example, in soybean photosynthesis increased at elevated CO_2 by an average of 39%, leaf area increased by 18%, and plant dry matter increased by 37% (Ainsworth et al., 2002). While increased CO_2 availability leads to a short-term increase in photosynthesis in C_3 species, longer exposures can lead to biochemical and molecular changes that result in a substantial decrease in photosynthetic capacity (Griffin and Seemann, 1996; Van Oosten and Besford, 1996; Ludewig and Sonnewald, 2000). This decrease in photosynthetic capacity has been associated with a decline in the activity of RuBisCO in many species (Sage et al., 1989; Long and Drake, 1992; Nie et al., 1995). In C_3 plants the response of photosynthetic CO_2 assimilation to leaf intercellular CO_2 concentration is governed by two distinct phases when measured under saturating light (Von Caemmerer and Farquhar, 1981). These are the carboxylation efficiency of RuBisCO and the regeneration rate of RuBP. High atmospheric CO_2 levels tend to increase the intercellular CO_2 in C_3 plants, which in turn increases the carboxylation efficiency of RuBisCO as the oxygenase reaction (photorespiration) is decreased. This is due to a greater amount of CO_2 being available as substrate for RuBisCO (Chaves and Pereira, 1992; Wullschleger et al., 1992; Hymus et al., 2001; Arena et al., 2005).

Since CO_2 is the substrate for the process of photosynthesis, it is highly likely that a change in the amount of available CO_2 will cause changes in this process which might

require rapid degradation of unnecessary protein, and/or necessitate post-translational processing of proteins that are required in greater abundance. An increase in the availability of CO_2 changes the control exerted by different enzymes of the Calvin cycle on the overall rate of CO_2 assimilation. This then alters the requirement for different functional proteins. Since increased CO_2 content decreases photorespiration in C3 plants, an increase in CO_2 availability will also decrease the requirement for enzymes and proteins involved in the photorespiratory flux. Furthermore, the decrease in RuBisCO protein at elevated atmospheric CO_2 (Nie et al., 1995) indicates that the expression and turnover of this protein might involve changes in the proteolytic mechanism of the plant cell. This change may be especially evident when plants are switched from an ambient atmosphere to a CO_2-enriched atmosphere, and may involve the selective degradation of RuBisCO protein by RuBisCOlytics. Developmental and environmental signals may cause changes in the quantity and quality of specific proteins in the choloroplast. This could be regulated by proteases and chaperones. Although a limited number of plastid proteases are known (Sakamoto, 2006), a number of ATP-dependent proteases (such as Clp, FtsH and Lon) are considered major enzymes involved in degradation of proteins to oligopeptides and amino acids.

Growth at elevated CO_2 may lead to increased levels of soluble carbohydrate (Bowes, 1993; Drake et al, 1997) which may cause feedback inhibition of photosynthesis (Stitt, 1991). Usually carbohydrate that is synthesised in source tissues are transported to sink tissues. However, when carbohydrate synthesis rate exceeds the rate at which soluble sugars and carbohydrates can be exported, this causes a source-sink imbalance that has to be corrected (Farrar and Williams, 1991). The photosynthetic machinery responds by, amongst other things, altering the quantity and/or activity of RuBisCO (Gesch et al, 1998). A change in the abundance of RuBisCO could be regulated on transcriptional, translational, or protein turnover level (Webber et al., 1994). Increased CO_2 availability causes an earlier peak and then decline of RuBisCO activity and content during leaf expansion compared to controls grown at ambient CO_2 (Winder et al, 1992). While it's known that RuBisCO small subunit transcripts are affected by CO_2 availability (Winder et al., 1992), the level of regulation by translation and posttranslational turnover is complicated by the fact that photosynthetically competent Rubsico has a relatively slow turnover rate (Peterson et al., 1973). However, research has shown that RuBisCO is regulated at the transcriptional, posttranscriptional, translational, and/or posttranslational

levels, depending on developmental factors and environmental signals (Gesch et al., 1998). The response of rice leaves to increased CO_2 availability was shown to be dependent on the developmental stage of the leaf, with mature leaves responding more strongly than expanding leaves where photosynthesis is concerned. In general, photosynthesis was 25% to 30% greater in mature leaves and 20% to 24% greater in expanding leaves of plants grown under high CO_2. Some results illustrate, however, that there is no change in the turnover rate of RuBisCO due to CO_2 availability. The large decline in RuBisCO protein under these circumstances - up to 60% (Sage et al., 1989; Besford et al., 1990; Rowland-Bamford et al., 1991) is therefore not necessarily due to increased proteolysis, but more likely a result of decreased transcription.

Another potential effect of CO_2 enrichment on RuBisCO is a change in the activation state of the enzyme (Crafts-Brandner and Salvucci, 2000; Rogers et al., 2001). RuBisCO activity is regulated by RuBisCO activase and by the binding of inhibitors such as carboxy arabinitol-1-phosphate (CA-1-P; Salvucci and Ogren, 1996; Parry et al., 1997; Portis, 2003). These factors determine the rate of flux through the enzyme in any given time and govern its activation state. The activation state reflects the number of RuBisCO holoenzymes that are actively fixing carbon out of the total pool of RuBisCO present in the plant cell. Decreased RuBisCO activity following CO_2 enrichment may therefore arise from a decline in the activation state of this enzyme (Vu et al., 1983; Sage et al., 1988; Van Oosten et al., 1994; Crafts-Brandner and Salvucci, 2000). This response is rapid as activation state can be regulated within a matter of seconds (Bowes, 1993) and could provide a rapid and regulated switching off of the active sites of RuBisCO in response to CO_2 enrichment. RuBisCO activase is an ATP-dependent AAA+ protein (Neuwald et al, 1999) that facilitates the removal of sugar phosphates (such as CA-1-P or RuBP) from RuBisCO active sites. Since it is dependent on ATP and is inhibited by ADP, the ratio of ATP to ADP in the chloroplast affects activase activity, and hence activation state of RuBisCO (Robinson and Portis, 1989). Furthermore, the level of mRNA abundance and enzyme activity of carbonic anhydrase that facilitates diffusion of CO_2 from intercellular air spaces (Edwards and Walker, 1983) decreases during growth at elevated CO_2 in pea (Majeau and Coleman, 1996), cucumber (Peet et al., 1986), and bean (Porter and Grodzinski, 1984). However, it remains unchanged or even increases in tobacco (Sicher et al., 1994) and *Arabidopsis* (Raines et al., 1992).

1.4.4 Degradation of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO)

As described above, it is quite possible that changes in the environment, such as an increase in the amount of available CO_2, will require rapid acclimation by the photosynthetic machinery, including the key enzyme in this process, RuBisCO. Furthermore, the degradation of RuBisCO is often used as a model for the turnover of proteins in plants. While over 50% of the protein content of green leaves is made up of this one enzyme (Fischer and Feller, 1994; Spreitzer and Salvucci, 2002) the nature of the proteolytic enzymes that are involved in RuBisCO degradation has not yet been determined. RuBisCO is an essential component of photosynthesis and it also serves as a reservoir of nitrogen. The RuBisCO holoenzyme consists of 8 large subunits and 8 small subunits (Fig. 1.8). The large subunit is encoded by a single chloroplastic gene (Chan and Wildman, 1972; Ellis, 1981), while the small subunits are encoded by a small family of genes in the nucleus (Manzara and Gruissem, 1988; Rodermel, 1999).

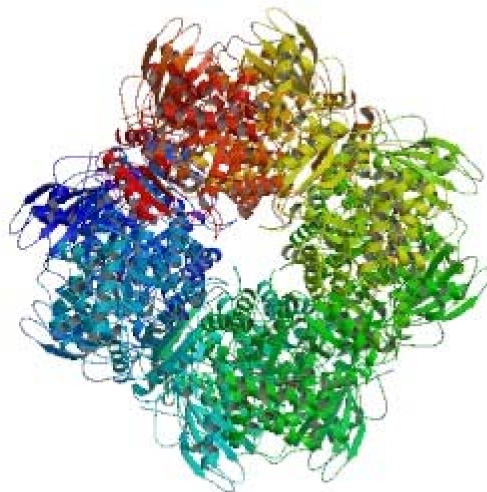

Figure 1.8 A graphical representation of RuBisCO. (Image from Protein Data Bank; http://www.msu.edu/~ngszelin/calvin_cycle_players.htm)

Unassembled small subunit proteins were selectively and rapidly degraded within the chloroplasts of the green alga, *Chlamydomonas reinhardtii*, when pools of large subunit were depleted (Schmidt and Mishkind, 1983). Intensive study has failed to characterise the complex network of processes that control RuBisCO breakdown. The protein can be degraded in intact chloroplasts by stromal proteases (Mitsuhashi et al., 1992; Desimone et al., 1996; Ishida et al., 1998; Adam and Clarke, 2002). A chloroplast-located

metallopeptidase has also been shown to degrade the RuBisCO large subunit (Bushnell et al., 1993; Roulin and Feller, 1998).

RuBisCO degradation can be initiated or accelerated by reactive oxygen species (ROS) in isolated intact chloroplasts (Desimone et al., 1996; Ishida et al., 1997). However, the RuBisCO protein, especially the large subunit, is also sensitive to degradation by vacuolar peptidases (Yoshida and Minamikawa, 1996). Considerable debate remains concerning the occurrence and function of degradation of the RuBisCO protein/peptides outside the chloroplast. A role of vacuolar proteases or other proteases present in cytosolic vesicles such as the ricinosomes or the lytic vacuoles has been postulated (Gietl and Schmid, 2001). During leaf senescence, the photosynthetic machinery is dismantled and chloroplasts are converted into gerontoplasts. Different models have been suggested for the degradation of chloroplast functions in senescing mesophyll cells (Krupinska, 2006): Plastids may be engulfed in the central vacuole by phagocytosis or by membrane fusion of plastid-containing autophagosomes with the vacuole. Considerable evidence suggests that chloroplasts (Minamikawa et al., 2001) and/or chloroplast-derived vesicles (Chiba et al., 2003) interact with the vacuole during senescence and facilitate rapid degradation of chloroplast proteins. Chloroplasts release vesicles from the tips of the stromules (Gunning, 2005; Fig. 1.9). These vesicles contain RuBisCO and other stromal material. These and other types of chloroplast-derived vesicles can also contain thylakoid-derived material. While the release of vesicles was originally considered to occur only during senescence when the plastid envelope is ruptured, recent evidence suggests that they are produced at all stages of development but their production is enhanced during senescence (Gunning, 2005). Accumulating evidence also suggests that RuBisCO and other stromal proteins can be degraded, at least in part, outside the plastid. The chloroplast-derived vesicles may be the vehicle through which this process is facilitated.

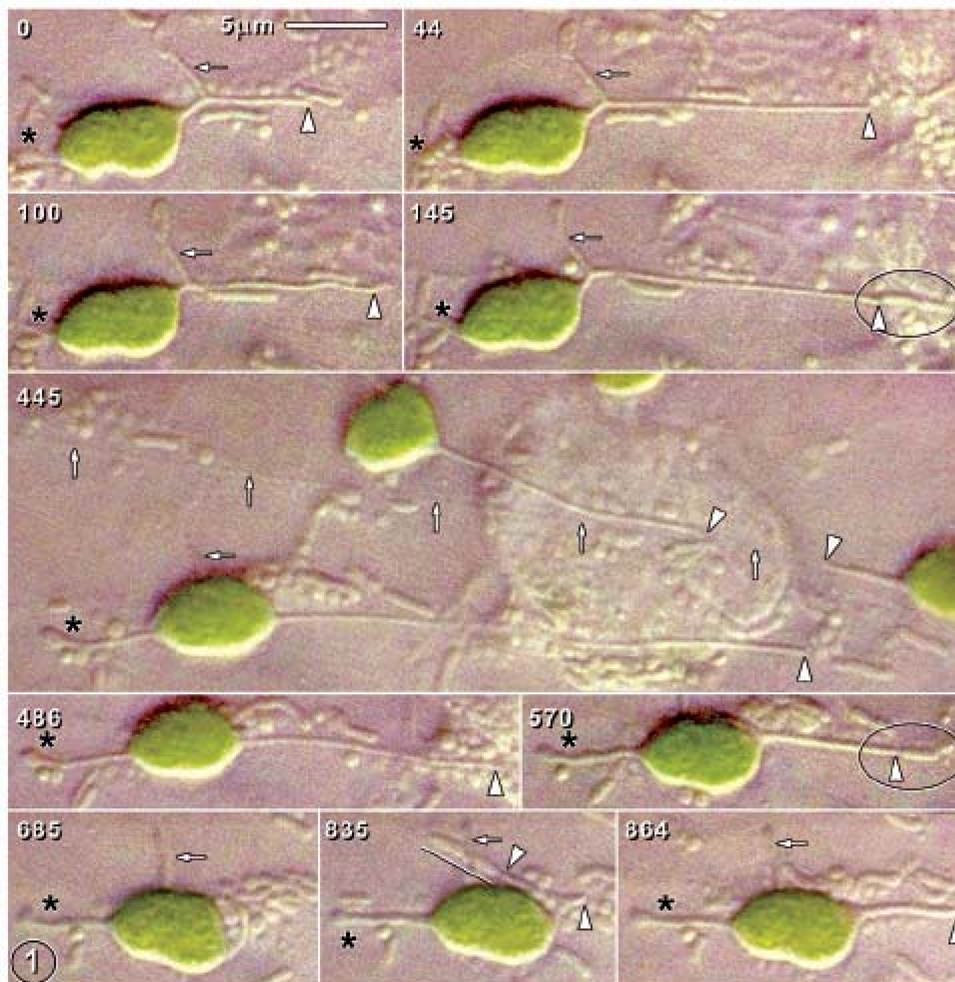

Fig. 1.9 Growth and retraction of stromules on *Iris unguicularia* chloroplasts (Gunning 2005). Elapsed times (seconds) are shown. Time 445 shows stromules on three chloroplasts, with a nucleus in the background. Vertical arrows mark a (presumed) actin cable along which organelles were streaming. The remaining images depict growth and retraction of the lower stromule, which also lay along a track of cytoplasmic streaming. Stromule tips are marked by arrowheads in each image. This chloroplast possessed multiple stromules. In addition to the main one there was a short stromule (asterisk) pointing in the opposite direction, and a branch (horizontal arrows). Both of these had terminal lobes, flattened close to the cell surface.

1.5 Increased CO_2 availability as an environmental signal for plant metabolic change

While high CO_2 can inhibit the maximal rate of photosynthesis (especially in C3 plants), effects are highly variable such that no general single high CO_2 response can be described (Sage et al., 1989). Studies on the effects of CO_2 enrichment on C_4 species has yielded rather mixed results. An enhancement of photosynthesis was found in some studies (Le Cain and Morgan, 1998; Wand et al., 2001) but acclimation and down-regulation were observed in others (Greer et al., 1995; Ghannoum et al., 1997; Walting and Press, 1997).

However, some C_4 species can benefit from CO_2 enrichment in terms of carbon gain (e.g. Ghannoum et al., 1997; 2001; Wand et al., 1999; 2001). For example, while growth at a high CO_2 level had little effect on photosynthetic capacity in *Paspalum dilatatum* leaves (von Caemmerer et al., 2001; Soares et al., 2008), high CO_2 –grown plants had double the total biomass of plants grown in air (Soares et al., 2008).

It is generally accepted that an increased CO_2 content in the atmosphere will have a fertilizing effect on plants (especially C_3 plants), as it will alleviate the CO_2 limitation on photosynthesis. To this effect, increased CO_2 availability is not considered to be an abiotic stress factor. This perspective is supported by results that show increased resource use efficiency in plants grown under elevated CO_2. In particular increased water, light, and nitrogen use efficiencies have been observed in plants grown at elevated CO_2 when compared to plants grown at ambient CO_2 levels (Drake et al., 1997). These plants also showed improved resistance to environmental stresses such as drought, chilling or air pollution (Boese et al., 1997; Hsiao and Jackson, 1999). It has been shown that growth CO_2 concentration can affect stress susceptibility in leaves of poplar trees. Elevated CO_2 levels protected leaves from stress-induced decrease in photosynthesis induced by cold stress or paraquat under hight light conditions (Schwanz and Polle, 2001). Growth under elevated CO_2 concentration improves the internal availability of carbon, thereby, providing better supply of stressed plants with substrates for detoxification and repair (Carlson and Bazzaz, 1982). While enhanced CO_2 increases carbon allocation, especially to roots (Bazzaz, 1990), it also enhances overall plant development and senescence in several species (Rogers et al., 1994). Increased CO_2 availability can also accelerate flowering and increase flower and fruit weight (Bazzaz, 1990; Deng and Woodward, 1998), although the effects of CO_2 on flowering and seed output of wild species vary strongly between species (Jablonski et al., 2002). Even though it was observed that CO_2 enrichment enhances senescence, the decrease of chlorophyll levels general associated with senescence is not always observed under these circumstances, where cholorphyll content increases, decreases or stays unchanged under elevated CO_2 (Vu et al., 1989; Heagle et al., 1993; Mulholland et al., 1997; Lawson et al., 2001; Bindi et al., 2002; Prins et al, 2008). Early senescence (indicated by premature yellowing and a decrease in chlorophyll content) was observed in the leaves of CO_2-enriched sweet chestnut seedlings. However, this response was associated with nutrient dilution caused by the rapid growth of seedlings, and this may have played a role in the early senescence of the plants. It has

been hypothesised that leaf senescence may be triggered earlier due to the different effects of an increase in CO_2 availability, especially during grain filling due to an increased grain nutrient sink capacity (Wingler et al., 2006). In contrast, it has been hypothesised that the increased C/N ratio in species that show increased photosynthesis rate in elevated CO_2 may lead to delayed autumnal senescence (Herrick and Thomas, 2003). Delayed senescence has been observed in populus species grown with CO_2 enrichment (Taylor et al, 2008).

1.6 Effects of CO_2 enrichment on plant morphology and stomatal patterning and function

Besdies changes in photosynthesis on a molecular level, plants grown at high CO_2 can also show changes in whole plant morphology. One example is the decreased shoot/root ratios that result from the acclimation of source-sink processes to increases in carbon gain as a result of higher rates of photosynthesis (Ghannoum et al., 1997; 2001; Walting and Press, 1997). Moreover, CO_2 availability has a strong influence on stomatal patterning and the dorso-ventral organisation of leaf structure and composition/activity (Taylor et al., 1994; Croxdale, 1998; Masle, 2000; Lake et al., 2001; Martin and Glover, 2007). Photosynthetic responses to changes in CO_2 availability may be connected at least in part to changes in stomatal conductance and/or stomatal density (Woodward, 1987; Boetsch et al., 1996). Early studies indicated that stomatal density decreased with increasing CO_2 concentrations (Woodward, 1987; Penuelas and Matamala, 1990; Lin et al., 2001). However, while a comparison of a hundred different species revealed a wide range of stomatal density responses to CO_2 enrichment, there was an average reduction in stomatal density of 14.3% (Woodward and Kelly, 1995). This decrease was independent of taxonomy, growth form, habitat, or stomatal distribution. These authors found that amphistomatous leaves showed greater CO_2–dependent changes in stomatal densities than hypostomatous leaves when grown in controlled environments between 350 and 700 $\mu l\ l^{-1}$ CO_2 (Woodward and Kelly, 1995). In particular, maize plants grown from 340 to 910 $\mu l\ l^{-1}$ CO_2 showed a 26% reduction in stomatal density.

It has been suggested that high CO_2-dependent decreases in stomatal density confer a selective advantage because of improved water use efficiencies (Hetherington and Woodward, 2003). A study on CO_2 enrichment in sorghum, which is a C_4 species like maize, showed that plants grown under Free-Air CO_2 Enrichment (FACE) conditions with

ambient plus 200 µl l^{-1} CO_2 had higher water use efficiencies (Conley et al., 2001). This increase was greater for plants subjected to a drought treatment than those that were well watered. Sorghum plants subjected to drought showed a 19% increase in water use efficiency based on grain yields, compared to 9% in the well-watered plots (Conley et al., 2001). However, whole plant biomass was increased by similar amounts (16% and 17% in wet and dry plots, respectively) suggesting that the increased water use efficiency effect was accompanied by altered assimilate partitioning between organs rather than effects on total carbon gain. These results suggest that C_4 species like sorghum and maize might reap additional benefits from future environments that are CO_2-rich and drought-prone. In contrast, a study looking at 48 accessions of *Arabidopsis thaliana* (a C_3 species) showed no clear trends in these responses (Woodward et al., 2002).

While the nature of the high CO_2 effect varies between species it is widely accepted that CO_2 levels influences stomatal density and patterning (Larkin et al., 1997; Lake et al., 2002). The CO_2-signalling pathways that orchestrate these changes in leaf structure and composition responses remain poorly characterised (Gray et al., 2000; Ferris et al., 2002) but signals transported from mature to developing leaves are considered to be important regulators of such responses (Coupe et al., 2006; Miyazawa et al., 2006). Hence, the CO_2 levels in the atmosphere are considered to be detected primarily by mature leaves. The CO_2 signal, which is then transmitted to the young, developing leaves, modulates stomatal development in a way that is independent of the CO_2 content experienced by the young leaves (Lake et al., 2001).

1.7 Concluding statement and research objectives

The global climate of the future will be much more variable than it is today. To ensure the sustainable production of crops in this future scenario, it is essential to have a much more comprehensive understanding of how plants perceive and respond to changes in their growth environment. The following study was undertaken in order to obtain an improved understanding of plant responses to a changing environment particularly with regard to the role of proteases in metabolic change and senescence triggered by developmental and environmental cues. The first focus of this thesis was the role of cysteine proteases in leaf protein turnover during development and abiotic stress. The effect of constitutive expression of a cysteine protease inhibitor, oryzacystatin I, was studied in tobacco with respect to development and cold stress tolerance. The second focus of this thesis was an

investigation into the effects of CO_2 enrichment on maize leaf transcriptome, physiology, photosynthesis, metabolism, and protein turnover

The hypotheses that formed the foundation for the following study were formulated as follows:

Constitutive expression of the cysteine protease inhibitor OC-I in tobacco alters plant development and protects photosynthesis against dark chilling (Van der Vyver et al, 2003). These plants can therefore be used to explore the stress-induced mechanisms of protein turnover that are regulated by cysteine proteases.

Hypothesis 1: Exogenous OC-I protects RuBisCO from degradation by endogenous proteases that function during development and cold stress.

Atmospheric CO_2 is a major component of climate change that influences plant morphology and metabolism. CO_2 affects many aspects of leaf biology from photosynthesis, sugar metabolism, and the expression of sugar metabolism-related genes to protein content and composition, stomatal density and patterning.

Hypothesis 2: Maize will respond to growth with CO_2 enrichment by acclimation in leaf biology underpinned by changes in gene expression.

Growth with CO_2 enrichment leads to early leaf senescence in some species. Comparisons of the leaf transcriptome at different developmental stages in maize plants grown in air and with CO_2 enrichment might be predicted not only to reveal developmentally regulated proteases and protease inhibitors but also identify those that were preferentially influenced by CO_2-dependent signals.

Hypothesis 3: Changes in plant metabolism due to CO_2 enrichment and development involves changes in the expression and/or activity of proteases and protease inhibitors.

The study was undertaken using two plant species, maize and tobacco. Tobacco was chosen for the analysis of the effects of the OC-I transgene on plant growth and development because OC-I –transgenic tobacco plants had already been generated in the laboratory. While a preliminary characterisation had indicated that plant growth and development were affected by the expression of the transgene, no detailed analysis of

effects on protein composition or turnover had been performed. These plants were therefore an ideal and readily available tool with which to study the role of cystatins.

Maize was chosen as it is the second most important commercial cereal crop world wide and it is grown widely in Southern Africa (Pingali, 2001; Pons, 2003). While there is an extensive literature on maize biology in general, relatively little information is on how maize will be affected by climate change particularly the increased levels of atmospheric CO_2 that will be present in the not too distant future. Maize genomics is well advanced (Keith et al., 1993) cystatin sequences have been identified (Abe et al., 1992; Abe et al., 1995; Abe et al., 1996; Yamada et al., 2000) and maize (corn) micro-array chips are commercially available. Maize also is a C_4 species and as stated previously our current knowledge about the effects of increasing atmospheric CO_2 levels on C_4 plants remains limited and much more information is required in order to be able to accurately predict how the forthcoming change in the earth's atmosphere with regard to greenhouse gases like CO_2 will modify the productivity of C_4 plants. This subject is important as well as extremely topical because it is predicted that maize will be used increasingly in bio-energy production as well as a food crop over the next 50 years. Literature reports on the effects of increased abundance of atmospheric CO_2 on C_4 species show large inter-specific variations. Further characterisation is therefore essential and urgent.

Maize and tobacco are chilling sensitive species. Since exposure to low temperature in the hours of darkness posses a problem to the productivity of both species and it is also a cause of crop losses in Africa, as in other parts of the world, this study focussed on the impact of the OC-I transgene on plant responses to dark chilling. In particular, the experiments focussed on how the expression of the exogenous protease inhibitor effects photosynthesis and RuBisCO turnover and so alters the tolerance of tobacco plants to dark chilling.

In addition to allowing a detailed characterisation of the effects of changes in two key environmental variables, CO_2 and temperature, on plant morphology and metabolism, these analyses also allowed an appraisal of the regulation of protein content and turnover in the natural senescence programme. A further aim was therefore the identification of potential senescence markers that can be used in future studies. The data obtained in the following investigations were used to compare the role of specific proteases and their

inhibitors in plant stress and senescence responses. While the original primary focus was the identification of novel cysteine proteases and their inhibitors, it soon became apparent that other proteases and inhibitors were also important in the plant responses to the variables under study. Two novel CO_2-modulated inhibitors were selected on this basis for further characterisation.

The specific objectives of the following study were:

1) To identify the mechanism through which exogenously expressed OC-I protects photosynthetically important proteins. It is expected that proteins such as RuBisCO might be protected from degradation by endogenous proteases.
2) To study the effect of exogenously expressed OC-I on senescence in tobacco plants. Since cysteine proteases play an important role in senescence, it is expected that constitutive expression of OC-I will delay senescence.
3) To characterize the effects of high CO_2 on whole plant growth, morphology and development in maize and to compare the effects of growth with CO_2 enrichment in young and old source leaves. It is expected that there will be little change in plant morphology since maize already experiences a high CO_2 environment on a molecular level. However, photorespiration might be further minimised in C_4 plants which might affect metabolism.
4) To characterize CO_2-dependent effects on maize leaf epidermal structure, in relation to photosynthesis and metabolism. It is expected that epidermal structure will change, based on previous results (Martin and Glover, 2007).
5) To study the effects of high CO_2 on the transcriptome of leaves at different positions on the stem in order to identify new genes that can be used as markers for senescence. Changes in CO_2 availability will send signals to cell nuclei as the plant acclimates to the different environmental conditions. This will be reflected in changes in transcript abundance between high CO_2-grown maize plants and those grown in air. It is expected that increased CO_2 might lead to early senescence due to increased leaf carbohydrate. This will provide new sequences that are linked to senescence.
6) To identify novel senescence- and high CO_2–regulated proteases and protease inhibitors. Changes in available CO_2 will necessitate changes in the photosynthetic system. It is expected that these changes will partially be effected by selective

proteolysis, which will result in changed expression or activity of proteases and their inhibitors.