CHAPTER 6:Discussion

The studies described in this thesis contribute to our current knowledge of the role that proteases and protease inhibitors play in plant growth, acclimation, and senescence. Firstly, a characterisation of the effects of an exogenous protease inhibitor (OC-I) expressed in tobacco showed that this inhibitor can effect plant morphology, senescence, and cold stress-induced proteolysis. Results of this part of the study show that a single transgene can possibly have multiple effects on development and stress tolerance, and also provide evidence for a mechanism of RuBiSCO proteolysis outside the chloroplast. Furthermore, the responses of maize to CO_2 enrichment were studied by analysing changes in gene expression, physiology, photosynthesis, and carbon metabolism in plants grown at 700 µl l^{-1} CO_2 compared to those grown in air. This part of the study furthermore characterised the effect of CO_2 enrichment on proteases and protease inhibitors by studying changes in gene expression as well as activity of proteases. The results support a role for sugar and redox signalling cascades in maize response to high CO_2.

6.1 The effect of OC-I expression on development and abiotic stress tolerance in tobacco (Chapter 2)

Previous results showing a protection of photosynthesis by exogenous OC-I expressed in tobacco plants (Van der Vyver et al., 2003) was the basis for the first chapter of this thesis. The objective of this chapter was to elucidate the possible mechanism by which the photosynthetic machinery is protected. The particular plants used in the previous study were already available in my research group and were used for these experiments. Endogenous phytocystatins perform a defensive role against biotic stress, such as insect and nematode attack (Liang et al., 1991; Zhao et al., 1996), and are also induced by abiotic signals, such as wounding or methyl jasmonate (Bolter 1993; Botella et al., 1996). The potential of exogenous OC-I to improve stress tolerance by protecting chloroplastic proteins was studied here. This study is the first to identify a novel role for the protection of RuBisCO and RuBisCO activase by the (presumably) cytosolic OC-I. This is an important result, since RuBisCO is the most abundant protein on earth, and an increase in protein content of crops will make them potentially more nutritious. Furthermore, this protection of photosynthetically important proteins may play a role in the observed tolerance of photosynthesis in OC-I expressing plants to cold stress. In this study an attempt was made to explain the protection of RuBisCO by suggesting a new model of

RuBisCO degradation. The degradation of RuBisCO is a controversial subject, with the main theories suggesting that RuBisCO is either mainly degraded inside the chloroplast with oligopeptides being exported for further degradation in the vacuole, or that RuBisCO is exported whole and degraded in vacuolar vesicles containing RuBisCO-degrading proteases (Feller et al., 2007). In this study immuno-localisation studies provides evidence that RuBisCO is exported from the chloroplast in vesicles for degradation in the cytosol. Any action of cysteine proteases in these vesicles and the protective effect of exogenously expressed OC-I still needs to be characterised. In particular, a follow-up study is being performed in order to identify the mechanism through which RuBisCO, OC-I, and the protease(s) it inhibits interacts. Preliminary results suggest that OC-I is present in vesicles in the cytosol. Furthermore, identification of the endogenous proteases that are inhibited by OC-I will be pursued.

6.2 The effect of CO_2 enrichment on photosynthesis and plant physiology (Chapter 3)

While the increasing concentration of CO_2 in the earth's atmosphere could have a positive effect on photosynthesis in C_3 plants, it was illustrated in this study that increased CO_2 availability does not alter photosynthesis in maize, a C_4 species. Photosynthesis and RuBisCO activity in C_4 species are not limited by CO_2 availability as it is in C_3 plants, due to the CO_2-concentrating mechanism employed by C_4 species. In addition to the lack of CO_2-induced changes in photosynthesis in maize plants grown at high CO_2, there was little or no significant change in the expression of photosynthesis-related transcripts. In *Arabidopsis*, increased availability of CO_2 is associated with an increased level of sugars, which leads to a hexokinase-mediated decrease in the expression of RuBisco SSU (Sun et al., 2002). In this study only a single probe set representing RuBisCO SSU was affected by CO_2 enrichment (by 30%), and only in young leaves. RuBiSCO SSUs are encoded by a multi-gene family that are expressed differentially at ambient CO_2 (Dean et al., 1989) and also respond differentially to CO_2 enrichment (Cheng et al., 1998), which could explain why only a single probe-set was identified. In agreement with the lack of observed changes in RuBiSCO SSU transcripts, there was no significant effect of CO_2 enrichment on the expression of hexokinase, which regulates the expression of this protein. While RuBisCO contributes only up to 30% of total soluble proteins in C_4 plants (Sugiyama et al., 1984), it represents, along with phosphoenolpyruvate (PEP) carboxylase, and pyruvate orthophosphate dikinase, nearly 50% of the soluble leaf proteins in C_4 plants (Sugiyama et

al., 1984; Sage et al., 1987; Makino et al., 2003). These proteins (PEP carboxylase and pyruvate orthophosphate dikinase), that also play important roles in photosynthesis, were similarly unaffected by CO_2 enrichment in young and old mature leaves (rank 12 and rank 3 respectively). Photosynthetic acclimation to elevated CO_2 in C_3 plants generally varies with leaf age, with mature leaves showing a large amount of acclimation (Bowes 1991; Stitt, 1991) while young leaves lack an acclimatory response (Nie et al., 1995). This lack of acclimation may in part be due to young leaves not yet being sufficiently developed as source leaves, and therefore not able to redistribute nitrogen away from RuBisCO until further development has occurred (Moore et al., 1999). However, since RuBisCO is not CO_2-limited in maize, and since all leaves used in this study were mature source leaves, this did not play a role. While CO_2 had no significant effect on the photosynthetic system, leaf rank had a significant effect with old mature leaves (rank 3) having a much lower rate of photosynthesis than young mature leaves (rank 12). This decrease in photosynthesis could be mediated by, amongst other systems, a hexokinase-dependent signal leading to the decreased expression of RuBiSCO SSU as indicated by a higher abundance of hexokinase transcripts in old mature leaves.

While increased CO_2 availability leads to increased crop yield and biomass, and decreased stomatal density in C_3 species, this was not observed in this study on maize, a C_4 species. Since maize grown at high CO_2 did not have significantly different rates of photosynthesis or carbohydrate content, this could be expected. Furthermore, while stomatal density did not decrease, stomatal area and epidermal cell density did. This is a novel finding that contrasts with previous research in which maize plants showed a reduction in stomatal density (Woodward and Kelly, 1995). Furthermore, maize grown at high CO_2 showed a significant decrease in protein and chlorophyll content in the leaves on the mid-section of the stem. This was not observed in the leaves towards the bottom or top of the stem. It is unknown which proteins are affected by CO_2 enrichment in the leaves on the mid-section of the stem, a question that requires further investigation. The old mature leaves (rank 3) also had a significantly lower soluble protein content compared to the young mature leaves (rank 12) despite CO_2 level at which plants were grown. This is possibly a decrease in the amount of RuBisCO, PEPcase, and pyruvate orthophosphate dikinase in these leaves which constitute the bulk of soluble protein in maize cells. A decrease in these proteins would support the observed decrease in photosynthesis in the old mature leaves. While photosynthesis measured per surface area was not significantly affected by CO_2

enrichment, photosynthesis rates measured on leaves on the mid-section of the stem were significantly higher per mg chlorophyll in leaves grown at high CO_2. Plants do not synthesise RuBisCO in excess of that which is required to maintain photosynthesis. In addition to this, plants do not synthesise chlorophyll in excess of that which is required for light absorption in order to drive CO_2 fixation by RuBisCO. If the decreased protein content of leaves grown at high CO_2 represents a decreased amount of photosynthesis-related proteins, such as RuBiSCO, this would explain the decrease in chlorophyll - the major pigment that absorbs light. The amount of light absorbed by the chlorophyll should not exceed the capacity of the plant to use the energy for the fixing of CO_2. However, since photosynthesis rates per leaf area is similar in leaves grown in air or with CO_2 enrichment; this suggests that another rate-limiting process has been affected. While C_4 plants employ a form of photosynthesis that decreases photorespiration, it is possible that maize leaves grown at high CO_2 experience even less photorespiration than those grown in air. This could possibly account for the more efficient photosynthesis rates measured per mg chlorophyll in maize leaves grown with CO_2 enrichment.

6.3 The effect of CO_2 enrichment on the maize transcriptome (Chapter 4)

While other studies on maize and CO_2 enrichment have identified major effects on signalling and metabolism-related sequences, this study identified a number of stress-related sequences which are affected by CO_2 enrichment. This is a new result not reported before. A lower abundance of oxidative stress-related sequences in leaves grown at high CO_2 was observed. This result was complemented by oxyblot analysis which showed that proteins extracted from leaves grown at high CO_2 experience less oxidative marking. Furthermore, two novel protease inhibitors, a putative serine protease inhibitor and putative Bowman-Birk inhibitor, were identified as being specifically controlled by changes in CO_2 availability. These sequences were more abundant in air-grown leaves than high CO_2-grown leaves – a result that has not been reported previously. Serine protease inhibitors (including the BBI-type proteins) not only play a role in the regulation of endogenous trypsin and chymotrypsin activity, but are also induced upon wounding and attack by pathogens and insects (Bowler and Fluhr, 2000; Budai-Hadrian et al., 2006). While the microarray study performed here shows that protease transcripts are not increased by CO_2 enrichment, protease activity (including serine protease activity) were enhanced in leaves grown at high CO_2. Decreased expression of serine protease inhibitors in leaves grown at high CO_2 could result in increased protease activity. Alternatively, the

increased abundance of a putative serpin and putative BBI in air-grown leaves might be a part of the apparent stress-related pattern of transcripts observed. This hypothesis is supported by the observation that pro-oxidants induce transcription of both these sequences, showing that the putative serpin and BBI may be regulated by an oxidative signal. In addition, transcript abundance of both putative serpin and putative BBI showed a positive correlation with the glucose content of leaves, suggesting that these sequences may also be regulated by hexose content. This finding also implies cross-talk between the hexose and oxidative stress signalling pathways – a process that has been illustrated previously (Roitsch et al., 2003; Tajima and Koizumi, 2006). However, only the putative serpin (which contains a pin2 domain) was induced by glucose, a result that has been observed in pin2 proteins (Johnson and Ryan, 1990). These results support previous evidence that an increase in atmospheric CO_2 can increase plant tolerance to biotic (Idso et al., 2000; Matros et al., 2006) and abiotic (Miller et al., 1998) stress.

6.4 The effect of developmental stage on the maize transcriptome (Chapter 5)

Developmental stage affected a large number of proteases, protease inhibitors, and uibiquitin-related sequences as measured by microarray analysis. In particular, ubiquitin-related proteolysis seems to be favoured in old mature leaves based on expression of sequences involved in this system of proteolysis. Seven serine protease-type sequences were affected by developmental stage, including subtilases, serine carboxypeptidases, and a LON protease. The subtilase transcripts were more abundant in young mature leaves, indicating a possible role for these proteins in maize leaf development or regulation of stomatal density. The LON protease mRNA was more abundant in old mature leaves than young mature leaves, where it presumably functions in the regulation of protein fidelity in mitochondria based on this function ascribed to LON proteases (Janska, 2005). All cysteine proteases identified by microarray study as differentially expressed due to differences in leaf rank were more highly abundant in young mature leaves than old mature leaves. However, two cysteine protease inhibitor sequences were differentially regulated between young and old mature leaves, with one probe set being more highly abundant in young leaves, and another being more highly abundant in old leaves. In addition, a subtilase inhibitor sequence was less abundant in young mature leaves compared to old mature leaves. These sequences together provide a pool of potential markers for developmental change. In particular, subtilase and subtilase inhibitor sequences might prove valuable to this effect.

Changes in transcript abundance associated with phytohormone response or synthesis in maize leaves of different ranks on the stem showed that there is a differential response, but that CO_2 enrichment does not necessarily affect this developmentally regulated response. Transcripts associated with the synthesis of ABA (terpene synthase) was especially highly abundant in rank 12 leaves compared to rank 3. As ABA plays a role in the induction of stress-related genes (especially protease inhibitors) this may indicate a signalling role for ABA in this capacity. A further phytohormone that showed differential regulation based on leaf rank was auxin. Transcripts associated with this hormone was, in general, higher in rank 12 than rank 3, indicating a possible role in cell elongation and division and the delay of senescence in young, mature leaves (rank 12).

6.5 Conclusion

In conclusion, the original hypotheses as stated in Chapter 1 can be evaluated as follows:

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

In this study it was observed that OC-I expression in tobacco alters development and protects photosynthesis by interacting with unknown endogenous proteases. Expression of the transgene leads to changes in the degradation of RuBisCO and, possibly, RuBisCO activase. RuBisCO was identified in vesicular bodies in the cytosol, supporting a new hypothetical mechanism for RuBisCO degradation outside the chloroplast. These results support the hypothesis and address objectives 1 and 2 as stated in Chapter 1

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

In this study it was observed that increased CO_2 availability causes acclimation of photosynthesis in maize leaves so that photosynthesis rates are not significantly different from those measured in air-grown leaves. Carbohydrate metabolism is also not significantly affected. Transcripts related to photosynthesis and sugar metabolism are, accordingly, not significantly affected. However, epidermal characteristics, protein content, and chlorophyll content are significantly changed. These results support the stated hypothesis and address objectives 3, 4, and 5 as listed in Chapter 1.

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

In this study it was observed that growth with CO_2 enrichment does not affect the time of flowering. Since flowering and fruit development is used as an indicator of the onset of senescence (Noode´n et al., 1997), this shows that CO_2 enrichment does not enhance senescence in maize. While protease activity is enhanced in leaves grown at high CO_2 based on in-gel activity assays, expression of protease genes are not affected according to microarray results. Furthermore, the expression of a large number of proteases and protease inhibitors are affected by developmental stage. Two putative serine protease inhibitors (a serpin and a BBI) were specifically affected by CO_2 enrichment, and appear to be regulated by oxidative and hexose signals. These results support hypothesis 3 and address objective 6 as stated in Chapter 1.

6.6 Future work

The study on the protection of photosynthetic proteins by exogenously expressed OC-I provide exciting results that pave the way for further investigation. The mechanism by which RuBisCO is degraded *in planta* is of particular interest. Most importantly, the interaction between OC-I and the endogenous protease(s) that it inhibits, must be characterised. For this co-immunoprecipitation may be performed. Antibodies that recognise the OC-I protein can be included in plant extract prepared from OC-I-expressing tobacco. The antibody binds to the OC-I protein and can be precipitated using protein-G or protein-A sepharose which binds most antibodies. Any proteins or proteases that interact with OC-I will be co-immunoprecipitated in this way. The precipitated complex can then be studied by electrophoresis, mass spectrometry, or sequencing. Furthermore, the cellular localisation of the interaction between OC-I and endogenous proteins is currently being studied by *in situ* immunolocalisation studies.

This also study paves the way for further research to elucidate the effect of increased CO_2 availability on C_4 plants. More specifically, the effect of CO_2 enrichment on the maize proteome is being studied at present to determine regulation of the high CO_2-response on translational level. Differences in the quantity of a number of proteins have already been identified. These proteins will be identified by mass spectrometry or sequencing. Furthermore, the effect of CO_2 enrichment on RuBisCO and RuBisCO activase should be studied by Western blot analysis to determine whether these proteins are decreased in

leaves on the mid-section of the plant stem. Measurement of RuBisCO activity in these leaves could also provide insight into the acclimation of maize photosynthesis to high CO_2. Rubisco activity can be measured using the radioactive method described in this thesis. The putative serpin and BBI identified as being specifically affected by CO_2 enrichment have to be characterised with respect to function, in order to confirm their identity. These proteins should be expressed as fusion proteins and used in activity assays such as zymograms or biochemical assays such as those described in this thesis. Furthermore, the effect of constitutive expression of these genes in a model plant, such as tobacco or Arabidopsis might provide further insight into their function during stress.