CHAPTER 5: CO_2 enrichment influences both protease and protease inhibitor expression in maize

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5.1 Abstract

Proteolytic enzymes are essential for protein turnover and hence the ability of cells to respond to changing environmental conditions. Since one of the focuses of this study was to identify changes in the expression and activity of proteases and their inhibitors in response to development or CO_2 enrichment, the following studies were performed. Expression of protease inhibitor and protease-related transcripts were studied, and protease activities were investigated using biochemical techniques. Shoots contain high activities of both serine and cysteine proteases. Leaf protease activities were greatly increased by growth with CO_2 enrichment. Transcriptome analysis was performed on young and old source leaves of maize plants that had been grown for 8 weeks under either 350µl l^{-1} (low) or 700µl l^{-1} (high) CO_2. However, relatively few protease transcripts were modified by CO_2 in young and old source leaves. Growth at high CO_2 favours decreased source leaf cysteine proteases and increased cystatins.

5.2 Introduction

Growth with CO_2 enrichment causes extensive acclimation of photosynthesis involving down-regulation of carbon assimilation and up-regulation of carbohydrate synthesis and respiration. This massive reorganization of metabolism requires specific expression and regulation of proteases (Schaller, 2004; Trobacher et al., 2006). These are involved in the selective breakdown of regulatory proteins and enzymes by the ubiquitin/proteasome pathway and also in the post-translational modification of proteins by limited proteolysis for protein assembly and subcellular targeting. However, while it is widely recognized that the serine, cysteine, aspartic, metalloproteases and metacaspases are intricately involved in many aspects of plant growth and development (Mitsuhashi and Oaks, 1994; Xu and Cye, 1999; Bozhkov et al., 2005; Sanmartín et al., 2005), little attention has been paid to the

responses and regulation of these major proteolytic enzymes by high CO_2. CO_2 enrichment may lead to early senescence and changes in the later stages of programmed cell death through accumulation of sugars (Paul and Pellny, 2003). Since senescence is regulated by proteases (especially cysteine proteases) and their inhibitors (Solomon et al., 1999; Wagstaff et al., 2002; Belenghi et al., 2003; Okamoto et al., 2003), this study aimed to identify high CO_2–responsive proteases and protease inhibitors. To this effect, the transcriptome of young and old source leaves in maize plants grown to maturity at either 350 or 700µl l^{-1} CO_2 was studied.

5.3 Materials and Methods

All methods were performed by A. Prins, unless otherwise indicated.

5.3.1 Plant material and growth conditions

Maize (*Zea mays* hybrid H99) seeds were germinated on moistened filter paper. Batches of seedlings were harvested at 6 days for analysis at this point. Each seedling was separated into seed, shoot and root for analysis. Other batches of seedlings were transferred to compost in 8.5l volume (25cm diameter) pots, in controlled environment rooms (Sanyo, Osaka) where atmospheric CO_2 was strictly maintained at either 350µl l^{-1} or at 700µl l^{-1}, with a 16-h photoperiod with light intensity of 800µmol m^{-2} s^{-1}, temperature of 25ºC (day)/19 ºC (night), and 80% relative humidity as described by Driscoll et al. (2006). At 8 weeks the following parameters were measured in leaf 5.

5.3.2 Protein quantification

Protein was quantified as described in Chapter 2 using Bradford reagent.

5.3.3 Proteolytic detection in plant extracts

i) Azocasein assay

Cysteine protease activity was measured according to Reichard et al. (2000) and Michaud et al. (1995) with modifications.

The assay was optimised for use with maize plant extracts prepared from seeds (dry, imbibed and germinated), seedlings (3-6 days post imbibition), young shoots and mature leaves. The assay was tested for linearity with increasing time (Fig. 2.4 A), substrate (Fig.

2.4 B), and extract (Fig. 2.4 C). In general, fresh plant material was extracted by grinding it in a mortar with a pestle on ice, in cold extraction buffer (0.1M citrate phosphate buffer pH 5.5, 0.1% Triton X-100) at a ratio of 1:2 (w/v), with the addition of a small amount of acid washed sand (Sigma, UK). Dry seeds were first ground in a coffee grind (Krups model 203-42 coffee grind) before extraction over ice. A small weight to extraction buffer ratio was used in order to obtain highly concentrated samples. Samples were centrifuged at 12 000rpm for 10min at 4°C and the supernatant transferred to a fresh tube for use in the assay. Protein concentration was determined as described, using Bradford reagent, and in general approximately 500µg protein was used to assay extract obtained from seeds, while approximately 300µg protein was used to assay extract obtained from seedlings. Reactions were set up in duplicate by adding 100µl sample (containing 300-500µg) protein to 10µl extraction buffer and 90µl reaction buffer containing azocasein (1%) in 0.1M citrate-phosphate buffer pH 5.5 and 5mM DTT. Samples were incubated for 0-10min at 37ºC, after which the reaction was stopped by adding 60µl TCA (20%). The tubes were allowed to stand for 30min at room temperature after which they were centrifuged at 14 500 rpm for 5min. The supernatant (150µl) was mixed with 1 volume (150µl) of 1N NaOH and the absorbance of the released dye measured at 436nm in a microplate reader. Values obtained at 0min were subtracted from values obtained at 10min to obtain the change in A_{436} caused by cysteine protease activity.

The contribution of specific proteases to the detected protease activities was determined by pre-incubating samples for 10min at 37°C with specific protease inhibitors. To test for serine protease activity, 2µl extraction buffer was replaced with 2µl 100mM PMSF (final concentration 1mM); to test for cysteine protease activity, 10µl extraction buffer was replaced with 10µl 1mM E64 (final concentration 100µM); to test for metalloprotease activity, 4µl extraction buffer was replaced with 4µl 100mM EDTA (final concentration 2mM).

To test the linearity of the assay over time, the increase in A_{436} obtained by incubating extract obtained from seedlings 3 days post imbibition (380µg soluble protein) at 15min intervals over one hour was determined (Fig. 5.1 A). To test the linearity of the assay with increasing amount of substrate (Fig. 5.1 B), extract obtained from seedlings 5 days post imbibition (340µg protein) was incubated with 0-0.8mg azocasein for 20h to obtain

complete hydrolysis. To test the linearity of the assay with increasing amount of extract (Fig. 5.1 C), extract obtained from seedlings 7 days post imbibition was diluted to obtain total soluble protein content of 20-100µg protein and assayed as described above.

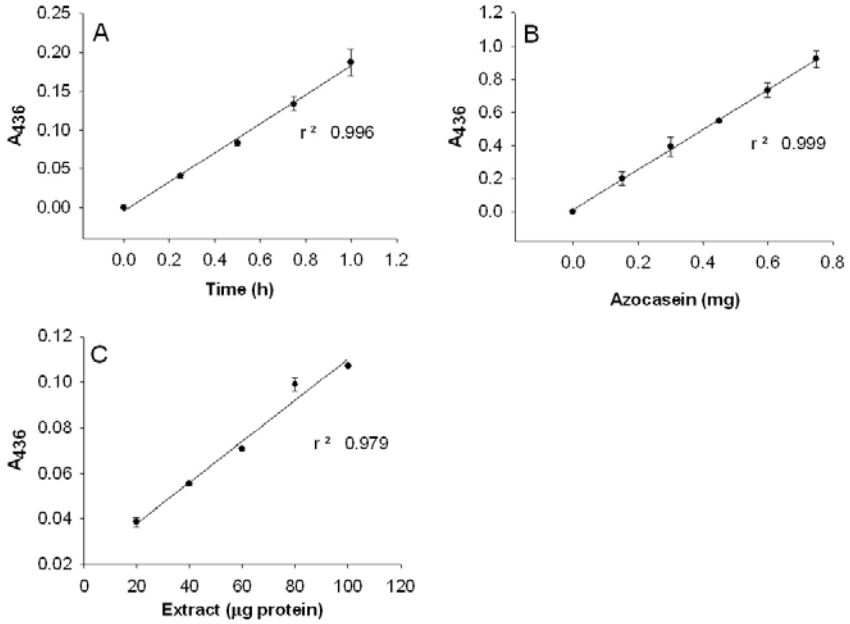

Figure 5.1 Optimisation of azocasein assay with respect to time (A), substrate (B) and extract (C).

ii) In-gel protease assay

In-gel detection of protease activity was done according to Michaud et al. (1993a). Protein samples were extracted in extraction buffer [0.1M citrate phosphate, 5mM DTT, 0.1% (v/v) Triton X-100] and centrifuged at 12 000 rpm for 10min at 4°C before determining protein concentration as described above. Extracts (containing 30µg protein) were then added to SDS PAGE loading buffer containing 62.5mM Tris-HCl (pH 6.8), SDS (2%, w/v), glycerol (10%, v/v), and bromophenol blue (0.001%, w/v). Samples were not boiled prior to gel electrophoresis and loading buffer did not contain β-mercaptoethanol to prevent destruction of secondary structure of proteins. Protein samples were separated on a 10% SDS gel containing gelatine (0.2%) in the resolving gel at 4ºC in 1x SDS PAGE buffer (Sambrook et al., 1989) until the blue front had reached the bottom of the gel. After electrophoresis, gels were placed for 30min in a 2.5% aqueous Triton X-100 solution to remove SDS and renature proteins. Gels were then rinsed with distilled water before being placed for 2-5h in a reaction solution consisting of 0.1M citrate phosphate buffer pH 5.5, 5mM DTT, and 0.1% Triton X-100 at 37ºC, with gentle shaking. After incubation, gels

were stained overnight in staining solution containing 0.25% Coomassie R-250 (w/v), 50% methanol (v/v), and 10% acetic acid (v/v). Gels were then destained in staining solution without Coomassie R-250 until white bands became visible.

To determine specific proteolytic activity, the protein extracts were preincubated in the presence of either 100µM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64; Sigma, UK) or 1mM phenylmethanesulfonyl fluoride (PMSF; Sigma, UK) at 37°C for 15min prior to the addition of sample buffer. E64 is an irreversible, potent, and highly selective inhibitor of cysteine proteases, while PMSF inhibits serine proteases, such as trypsin and chymotrypsin as well as some cysteine proteases (such as papain), although the inhibition of cysteine proteases is reversible by adding reducing agents such as DTT.

5.3.4 RNA extraction, purification, and analysis

RNA was extracted, purified, and analysed as described in Chapter 4 using Trizol reagent.

5.3.5 Micorarray hybridization

Microarray hybridization was performed using commercially available microarray chips (Affymetrix) at ArosAB in Denmark, as described in Chapter 4.

5.3.6 Microarray analysis

Microarray data analysis was performed by P. Verrier (Rothamsted Research, UK) as described in Chapter 4. Probesets were identified by translated homology search as described in Chapter 4.

5.4 Results

5.4.1 Protease activities

The tissue-specific protease activities of seeds, shoots and roots were examined in 6 day-old maize seedlings as well as in leaf 5 of 8 week-old plants (Fig. 5.2). Four well-defined activity bands were identified in the seed extracts (Fig. 5.2 B). These ranged in size from a high molecular weight (band i) to very low molecular weight (band iv). Band (i) was also present in the root, which additionally showed a large but very diffuse band of activity between bands (ii) and (iii). This diffuse band in roots may represent a single protease with high activity, or a number of proteases that all have similar molecular weights. The shoot and mature source leaf

both also showed a large diffuse area of activity between bands (ii) and (iii; Fig. 5.2 B), that, upon inspection, seemed to consist of a large number of distinct bands. The intensity of this diffuse area between bands (ii) and (iii) (Fig. 5.2 B) was greater in mature leaves than in shoots per µg protein, showing comparatively greater protease activity in mature leaves than young, developing leaves. This diffuse area was also enhanced in the leaves of the plants grown with high CO_2 compared to those grown in air (Fig. 5.2 C).

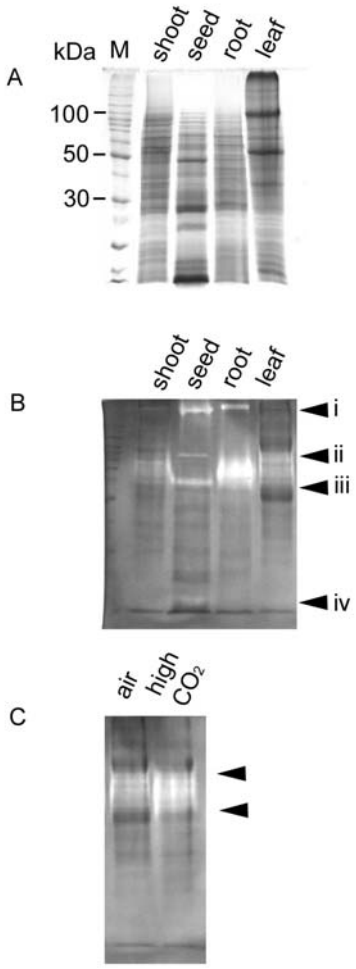

Figure 5.2. Protease activities in different maize organs. Tissue soluble proteins were identified on Commassie-stained gels loaded with equal protein (30 µg; A) and protease activities were detected by the in-gel assay (B and C). The gels in (B) had been loaded with different amounts of protein according to band intensity. The protein loadings were: 30µg shoot; 50µg seed; 15µg root; 30µg leaf. The gels in (C) had been loaded with equal leaf protein (30µg).

The inhibitory effect of PMSF on activity band (i), in shoot and seed extracts from 6 day-old seedlings, suggests that it comprises largely of serine proteases but the presence of PMSF plus DTT caused less suppression of band (i) activity in roots (Fig. 5.3 B) indicating that this band is represented by both serine and cysteine proteases. Seed

protease bands (ii) and (iv) were faint. They were absent in the presence of E64 but present in PSMF (Fig. 5.3 B), which implies that these bands could represent cysteine proteases, but not serine proteases. In contrast seed protease band (iii) was unaffected by E64 or PMSF. Similarly, neither E64 nor PMSF had any effect on the intensity of the diffuse zone of activity between bands (ii) and (iii) in roots, suggesting that this activity in roots arises largely from activity of proteases other than cysteine or serine proteases.

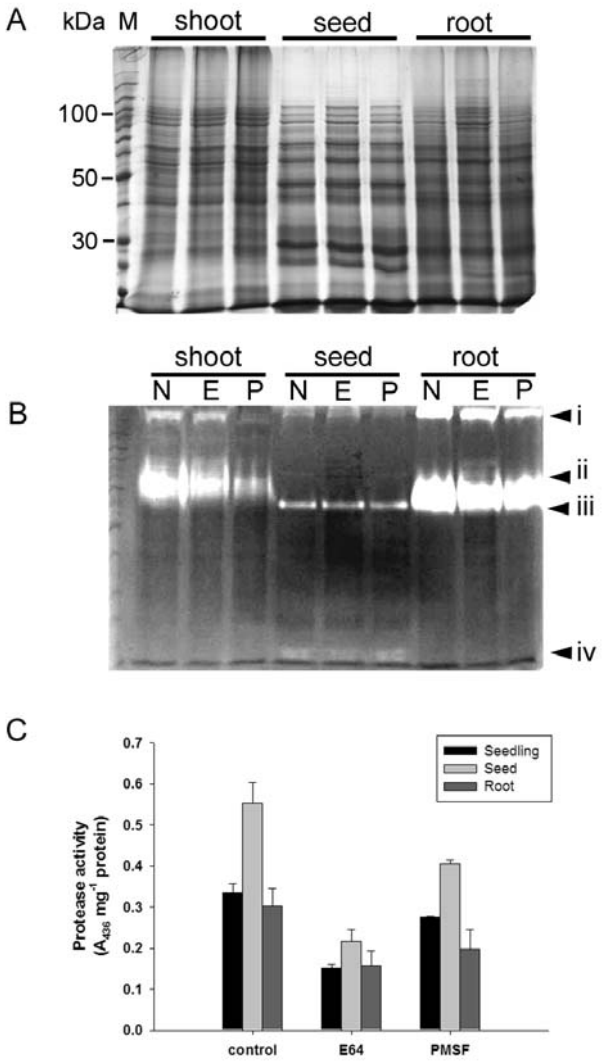

Figure 5.3 Characterization of protease activities in seed shoot and root. Tissue soluble proteins were identified on Commassie-stained gels loaded with equal protein (30µg; A) and protease activities were detected by the in-gel assay (B). The samples in (B) had been pre-incubated either in the absence of inhibitor (N) or in the presence of E64 (E) or PMSF (P). Gels had been loaded with 30µg protein in each case. Spectrophotometric analysis of protease activity (C) was performed on replicate samples to those in A and B.

The diffuse band of activity between bands (ii) and (iii) in shoots was diminished in the presence of E64 and PMSF, suggesting that this activity arises from a complex mixture of

different proteases (cysteine, serine, aspartic proteases and possibly others Fig. 5.3 B). Since this area also corresponds to the area affected by CO_2 enrichment in mature leaves, it is possible that the proteases up-regulated by CO_2 enrichment represent a mixture of different classes of protease.

Quantification of the protease activities, measured on a protein basis in the different tissues of germinating 6 day-old seedlings showed that seeds had the highest protease activities (Fig. 5.3 C). A substantial proportion (61%) of the seed protease activity was inhibited by E64. Similarly, about half of the total root protease activity was inhibited by E64 (Fig. 5.3 C).

5.4.2 Transcriptomic analysis

A preliminary analysis of the transcriptome data (Table 5.1) revealed that the abundance of two cysteine proteases and a chloroplast protease was decreased in the young source leaves of high CO_2 grown maize plants, although only by 21.5%, 18.1%, and 11.3% respectively. An aspartic protease was repressed by high CO_2 in the older source leaves by 17.7%. In contrast to the rather modest effect of CO_2 enrichment on protease transcripts, a large number of cysteine protease inhibitor (cystatin) transcripts were differentially regulated in the leaves of high CO_2-grown plants (Table 5.1). None of these transcripts differed in abundance by more than 25%. Hoever, a trend was observed whereby cystatins are induced in young mature leaves by CO_2 enrichment, while in old mature leaves they are repressed. Trypsin inhibitor transcripts were 31.9% higher in air-grown leaves than high CO_2-grown leaves. In contrast, transcripts encoding corn cystatin 1 were much higher in the leaves at elevated CO_2. Surprisingly, a wound-induced protein (WIP1) transcript, encoding a serine-type endopeptidase inhibitor, showed the greatest overall response to growth with CO_2 enrichment in young source leaves but not in old source leaves. The response of ubiquitin transcripts to CO_2 enrichment also varied with the stage of development, showing the greatest response in the old source leaves (Table 5.1).

Table 5.1 Protease and protease inhibitor transcripts that were differentially modified in response to CO_2 enrichment in maize source leaves. Transcript abundance was measured by microarray.

		(%change)	
Probe set	Gene title	Young source leaf	Old source leaf
Proteases			
Zm.7736.1.S1_at	Cysteine protease Mir2	-21.5%	3.0%
Zm.5987.1.A1_at	Cysteine protease	-18.1%	-10.5%
Zm.18435.1.A1_at	Protease Do-like 8, chloroplast precursor	-11.3%	1.3%
Zm.10845.1.A1_at	Nucellin-like aspartic protease	2.1%	-17.7%
Zm.5987.2.A1_at	Cysteine protease Mir3	-1.3%	9.9%
Protease inhibitors			
Zm.6656.1.A1_at	Trypsin inhibitor	-31.9%	-10.4%
Zm.14272.4.S1_x_at	Corn cystatin I	22.2%	-17.6%
Zm.14272.2.A1_a_at	corn cystatin I	20.2%	-8.5%
Zm.14272.5.S1_x_at	Corn cystatin I	20.0%	-13.5%
Zm.14795.1.A1_at	Putative cystatin	-15.2%	-6.9%
Zm.15278.1.S1_at	Protease inhibitor	12.8%	-1.2%
Zm.3347.1.A1_at	Putative cystatin	-10.0%	3.4%
Other			
Zm.186.1.S1_at	Wound induced protein	-120.3%	3.4%
Zm.3830.1.S1_at	Ubiquitin	1.6%	11.7%

5.5 Discussion

Growth with CO_2 enrichment had a pronounced effect on both source leaf protease activities and transcripts. However, high CO_2 also had a marked effect on the abundance of transcripts encoding protease inhibitors, particularly cystatins. Interestingly, high CO_2 led not only to marked decrease in the abundance of transcripts encoding a number of cysteine proteases but also to an increase in abundance of endogenous cystatin transcripts, particularly in young leaves. While the abundance of different proteases was not changed by a large percentage, there was a distinctive increase in observable protease activity due to CO_2 enrichment, as observed on activity gels. If the observed protease activity observed in leaves and shoots include, amongst other proteases, cysteine protease, this would suggest that high CO_2 causes modulation of cysteine protease activity at the level of translation or interaction with endogenous inhibitors. The protease inhibitor most significantly affected by CO_2 enrichment is a trypsin inhibitor, where the trypsin inhibitor was down-regulated by 31.9% in young source leaves. Trypsin inhibitors not only regulate endogenous proteases, but are regularly induced upon insect attack and therefore are characteristic of a biotic stress response. These results are in agreement with the results obtained in Chapter 3 where high CO_2-grown leaves had a lower abundance of stress-related transcripts, including two putative protease inhibitors.

In addition, there was a large decrease (120.3%) in the serine protease inhibitor, WIP1, in young source leaves due to CO_2 enrichment, which further confirms results obtained in Chapter 3, especially with respect to the specific regulation of serine protease inhibitors by CO_2. Regulation of protease activity in addition to abundance might be a key feature of this response.

This study is the first to identify the regulation of proteases and inhibitors by CO_2 enrichment. While protease transcripts are decreased (although in general, not significantly), protease activity is increased in leaves grown at high CO_2. In addition, the abundance of transcripts encoding protease inhibitors is generally differentially affected by CO_2 enrichment. However, the strong down-regulation of two stress-related protease inhibitors suggest that leaves grown in air experience a stronger stress-related signal than those grown with CO_2 enrichment.