



CHAPTER 3: Specification of adaxial and abaxial stomata, epidermal structure and photosynthesis to CO₂ enrichment in maize leaves

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3.1 Abstract

The results from Chapter 2 indicated a strong role for cysteine proteases in plant acclimation to cold stress. These proteases are known to play an important role in senescence. Since CO₂ enrichment could both enhance or delay senescence - dependent on species and development stage of plants - the question was asked whether an increase in CO₂ would affect senescence in maize, and hence proteases or protease inhibitors that regulate this process. In order to answer this question acclimation to CO₂ enrichment was studied in maize plants grown to maturity at either 350 or 700 μl l⁻¹ CO₂. In this part of the study, plants grown with CO₂ enrichment were significantly taller than those grown at 350 μl l⁻¹ but they had the same number of leaves. High CO₂ concentration led to a marked decrease in whole leaf chlorophyll and protein. The ratio of stomata on the adaxial and abaxial leaf surfaces was similar in all growth conditions, but the stomatal index was considerably increased in plants grown at 700 μl l⁻¹. Doubling the atmospheric CO₂ content altered epidermal cell size leading to fewer, much larger cells on both leaf surfaces. The photosynthesis and transpiration rates were always higher on the abaxial surface than the adaxial surface. CO₂ uptake rates increased as atmospheric CO₂ was increased up to the growth concentrations on both leaf surfaces. Above these values, CO₂ uptake on the abaxial surface was either stable or increased as CO₂ concentration increased. In marked contrast, CO₂ uptake rates on the adaxial surface were progressively inhibited at concentrations above the growth CO₂ value, whether light was supplied directly to this or the abaxial surface. These results show that maize leaves adjust their stomatal densities through changes in epidermal cell numbers rather than stomatal numbers. Moreover, the CO₂-response curve of photosynthesis on the adaxial surface is specifically determined by growth CO₂ abundance and tracks transpiration. Conversely, photosynthesis on the abaxial surface is largely independent of CO₂ concentration and rather independent of stomatal function.



3.2 Introduction

Stomata are the portals for gas exchange between the leaf mesophyll cells and the environment. They occupy between 0.5% and 5% of the leaf epidermis and are most abundant on the bottom or abaxial surface. Amphistomatous leaves, such as maize, have stomata on both sides. The pattern of the epidermal cells and abaxial/adaxial polarity of the maize leaf is established in the meristem and is subsequently maintained throughout leaf development (Juarez et al., 2004). It is not surprising, therefore, that the abaxial/adaxial polarity of maize leaves is genetically controlled. The abaxial surface receives and exchanges cell fate-determining signals with the adaxial epidermis. Several mutants involved in the regulation of this development have been described including the rolled leaf1 (Rld1-0), which shows partial reversal of polarity and adaxialization, and the leafbladeless1 (lbl1) mutant that has abaxialized leaves (Nelson et al., 2002). The RLD1 and LBL1 proteins are considered to act in the same genetic pathway to maintain the dorsoventral features of the leaf and to govern adaxial cell fate. These components appear to function upstream of members of the maize yabby family that act only after adaxial/abaxial polarity has been established (Juarez et al., 2004). In *Arabidopsis thaliana*, two related transcription factors, the R2R3 MYB proteins FOUR LIPS and MYB88, jointly restrict divisions late in stomatal cell formation (Lai et al., 2005).

The concept that stomatal structure and function has been honed through evolution to optimize the ratio of CO₂ uptake to water lost through photosynthesis, is now widely accepted. Species with the C₄ pathway of photosynthesis have further optimized CO₂ uptake processes to minimize water loss and photorespiratory CO₂ release. In maize leaves for example, CO₂ is absorbed in photosynthesis and CO₂ released in respiration at a ratio of about 17:1. As such, one hectare of maize in the field can remove about 22 tonnes of CO₂ from the atmosphere in a single growing season.

CO₂ is not only a passive substrate in gas uptake processes, but it is also involved in signal transduction processes that influence leaf structure and function. It is now established that long-distance signalling of information concerning CO₂ concentration is transmitted from mature to developing leaves (Lake et al., 2001) in such a way as to control absolute stomatal numbers and stomatal function (Lake et al., 2002; Woodward, 2002). Relatively few components of this signalling pathway have been identified. The *Arabidopsis* high

carbon dioxide (HIC) gene, for example, which encodes a putative 3-ketoacyl coenzyme A synthase, is a negative regulator involved in the CO₂-dependent control of stomatal numbers (Gray et al., 2000).

Extensive acclimation of photosynthesis to CO₂ enrichment is observed in plant species with either the C₃ or C₄ pathways of photosynthesis (Nie et al., 1995; Jacob et al., 1995; Tissue et al., 1993; Drake et al., 1997; Watling et al., 2000). Acclimation involves down-regulation of carbon assimilation pathways and up-regulation of processes using assimilate such as carbohydrate synthesis and respiration (Winzeler et al., 1990; Stitt, 1991; McKee and Woodward, 1994; Smart et al., 1994; Tuba et al., 1994; Nie et al., 1995). CO₂ enrichment-dependent carbohydrate accumulation is involved in the orchestration of gene expression (Stitt, 1991; Jang and Sheen, 1994; Van Oosten and Besford, 1996). Since sucrose and hexose-specific signalling mechanisms link source metabolism to nitrogen signalling and to hormone signalling pathways (Finkelstein and Lynch, 2000; Finkelstein and Gibson, 2002; Leon and Sheen, 2003), it is probable that sugar signalling is also involved in long-distance CO₂ signalling from mature to developing leaves. CO₂ enrichment induces changes in cell structure (Robertson and Leech, 1995; Robertson et al., 1995) and in whole plant morphology (Lewis et al., 1999, 2000). In sorghum, the increase in growth CO₂ from 350 to 700 μl l⁻¹ resulted in a marked decrease in the thickness of the bundle sheath and decreased CO₂-saturated rates of photosynthesis (Watling et al., 2000). There is little information in the literature concerning the effects of long-term CO₂ enrichment on maize leaf stomata structure/function relationships, particularly with regard to the abaxial/adaxial polarity of the leaf. The following experiments were therefore undertaken to investigate the acclimation of abaxial/adaxial morphology and photosynthetic function to CO₂ enrichment in maize

3.3 Materials and Methods

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

3.3.1 Plant material and growth conditions

Maize seeds (*Zea mays* variety H99) were obtained from Euralis (EURALIS Semences, Blois, France). For all experiments, plants were germinated in air in batches (12-14 plants per batch). The dry seeds were first immersed in deionised water and stirred for 2h at



room temperature. Seeds were placed separately in rows on moistened filter paper and placed in closed plastic containers at 24°C in darkness for 3-4 days to allow germination to occur. The germination rate was 96-100%. Individual germinated seeds were transferred to small pots (one per pot) containing a commercial peat/loam compost blend (Petersfield Products, Cosby, UK). This consisted of 75% medium grade peat, 12% sterilised loam, 3% medium grade vermiculite, 10% grit (5mm screened, lime free) with added nutrients [N (14%), P₂O₅ (16%), K₂O (18%), MgO (0.7%), B (0.03%), Mo (0.2%), Cu (0.12%), Mn (0.16%), Zn (0.04%), Fe (chelated) (0.09%)]. Pots were transferred to controlled environment cabinets (Sanyo 970, Sanyo, Osaka) or controlled environment rooms (Sanyo, Osaka) as illustrated in Figures 3.1 and 3.2, where atmospheric CO₂ levels were strictly maintained at either 350µl l⁻¹ or at 700µl l⁻¹ (Fig. 3.4). The plants were grown with a 16-h photoperiod at a light intensity of 800µmol m⁻² s⁻¹ (at leaf level) with a day/night temperature of 25°C /19°C, and 80% (v/v) relative humidity. When the roots started to appear at the base of the small pots, the plants were transferred to 8.5l volume (25cm diameter) pots. They were then grown to maturity (8 weeks; 12-13 leaf stage). Plants were irrigated twice daily and maintained in water-replete conditions throughout. Samples were harvested for assay at various stages of development as indicated in the text and figure legends.

Certain batches of plants were grown at 350µl l⁻¹ until the 5th leaf had reached the mid-emergence stage, at which point they were transferred to 700µl l⁻¹ CO₂. Just prior to transfer, the base of the 5th leaf was marked with tippex to identify the portion which had emerged at 350µl l⁻¹ CO₂.

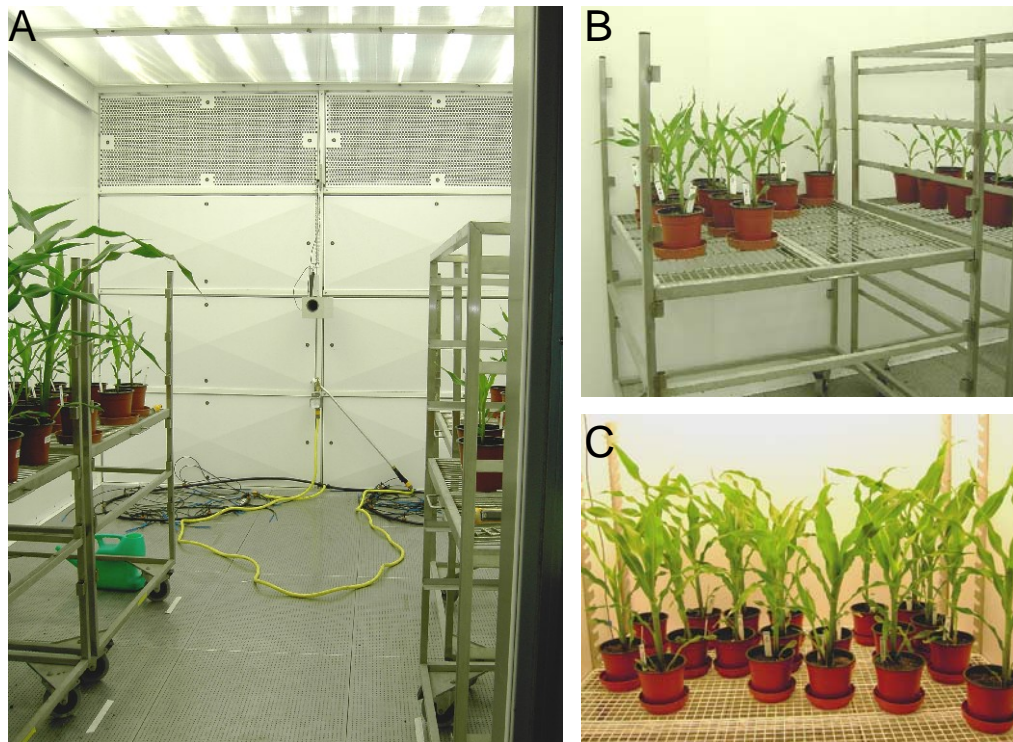


Figure 3.1 The controlled environment growth rooms and cabinets used in this study. Maize plants were grown either on trolleys in the controlled environment growth rooms (A, B) or on racks in the controlled environment cabinets (C), so that plant height could be adjusted to ensure uniform irradiance throughout development.

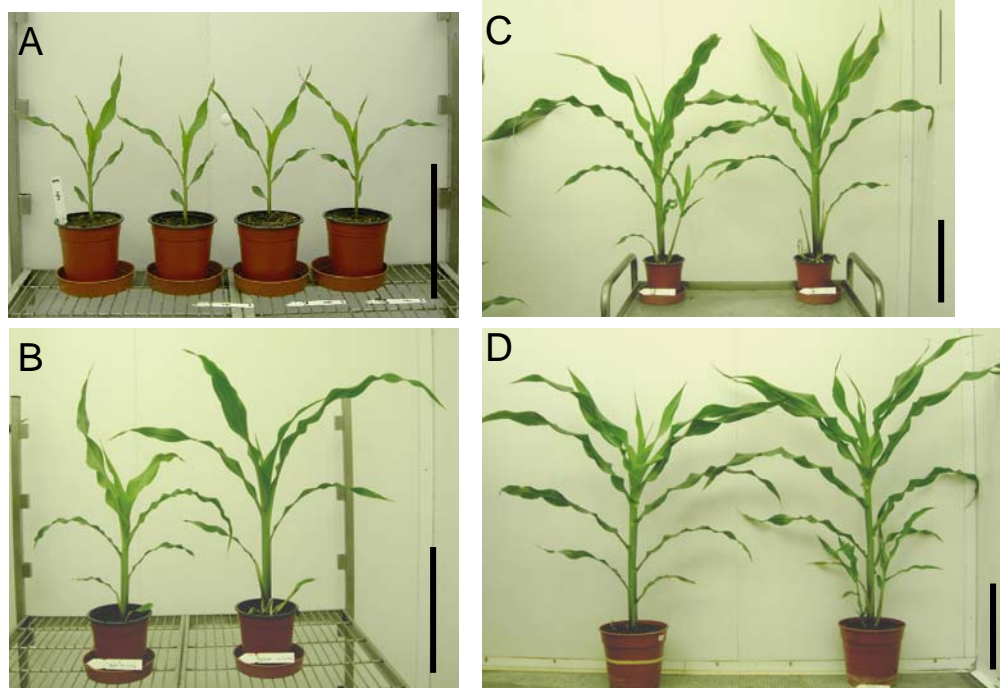


Figure 3.2 Relative low ($350\mu\text{l l}^{-1}$) and high ($700\mu\text{l l}^{-1}$) CO_2 phenotypes at different developmental stages. Plants are shown at the following stages: A) 9 days, B) 18 days, C) 25 days, and D) 32 days. In A, the first two plants (left) had been grown in air while the second two plants (right) had been grown with CO_2 enrichment. In B, C and D the plant

on the left had been grown in air while the plant on the right had been grown with CO₂ enrichment. (Scale bar = 24 cm).

3.3.2 Growth analysis

The following measurements were performed at the 12-13 leaf stage (8 weeks) from plants grown either in air or at high CO₂ (Fig. 3.4). In all experiments leaf phylogeny was classified from the base to the top of the stem, leaf one being at the bottom and leaf twelve at the top. Measurements were performed sequentially as follows:

i) Stem height

Stem height was measured from the base to the top of the stem with a ruler.

ii) Numbers of leaves, cobs, and tillers

Leaves were counted from leaf 1 to 12 as above. Although no standard fertilization procedures were applied, numbers of cobs and tillers were counted.

iii) Leaf weight

The fresh weight of each leaf was measured following excision. Total leaf fresh weights were determined on a standard laboratory balance.

3.3.3 Photosynthesis and related parameters

The following experiments were conducted on the 6th or 7th leaves of eight week-old plants. The same leaf segments were used for gas exchange and structural analysis. Measurements were made on either side of the mid-rib of individual leaves where the width was approximately 4cm, 24 and half cm from the leaf tip i.e. about half way from base to tip. Where plants had been exposed to a single CO₂ concentration during growth, the leaf area used for gas exchange measurements was also used for stomatal and epidermal characterisation. In leaves that had experienced growth in air for 18 days and then moved to a high CO₂ environment, two samples were taken (Fig. 3.3). The leaf part exposed to 350µl l⁻¹ CO₂ was sampled by taking a 7.5x3.5cm piece on the leaf tip-side, right next to the mark indicating the area of leaf that emerged in air. The leaf part exposed to 700µl l⁻¹ CO₂ was sampled by measuring 15cm from the mark (measured towards the leaf base), and taking a 7.5x3.5cm piece at this point on the leaf.

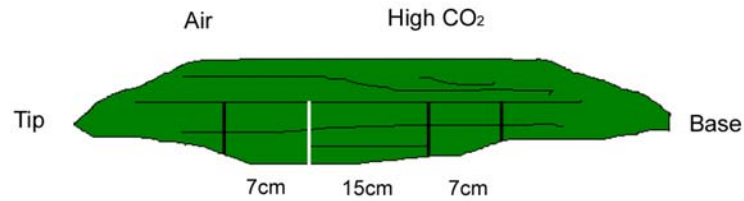


Figure 3.3 Leaf sampling to observe stomatal and epidermal characteristics in leaves exposed to air for 18 days and thereafter to high CO₂. Samples were taken next to the white mark, which indicates the leaf tip that emerged in air, and 15cm from this mark towards the leaf base, where the leaf had emerged in a high CO₂ environment.

i) Epidermal structure and stomatal patterning

Leaf pieces (7cm x 3.5cm) were harvested and epidermal tissue was stripped from the adaxial and abaxial surfaces using forceps. The epidermal peels were mounted in citrate phosphate buffer (0.1M sodium citrate, 0.1M sodium phosphate, pH6.5) and examined by light microscopy (Olympus BH-2, Olympus Optical Co. Ltd, Tokyo, Japan). The total area and numbers of stomata and epidermal cells were counted. At least 90 cells were measured from each of the digitised images from six sections using Sigma ScanPro photographic analysis software Version 5 (Sigma Chemical Co.). The digitised images were sent to E. Olmos (CEBAS-CSIC, Murcia, Spain) who performed the cell area and density measurements. The stomatal index was calculated as the number of stomata/ (number of epidermal cells + number of stomata) times 100, according to Salisbury (1927)

ii) Gas exchange, transpiration, and stomatal conductance

Gas exchange measurements were performed by S.P. Driscoll (Rothamsted Research) with assistance by A. Prins. Photosynthetic gas exchange, transpiration, and stomatal conductance was measured on attached leaves using an Infra Red Gas Analyser (model wa-225-mk3, ADC, Hoddesdon, Hertfordshire, UK). In these experiments specialized leaf chambers were used that allow simultaneous measurements of CO₂ assimilation and transpiration on each leaf surface independently. All experiments were conducted at 20°C with 50% relative humidity. The gas composition was controlled on each half of the chamber by a gas mixer supplying CO₂ and 20% O₂ and with the balance made up with N₂. The CO₂ response curves for photosynthesis were measured at 900–1000 μmol m⁻² s⁻¹ irradiance. Steady-state rates of CO₂ uptake were attained at each CO₂ concentration. The CO₂ level in the chambers was increased step-wise from 50 to 1000 μl l⁻¹.



3.3.4 Protein and chlorophyll quantification

i) Protein

For the leaf protein profile, a disk (9.62cm^2) was cut from each leaf at a distance 12-21cm from the leaf tip (where the leaf blade could accommodate the disk diameter of 3.5cm), frozen in liquid nitrogen and stored at -80°C until protein content was determined. Leaf disks were ground in ice-cold mortars with pestles, using liquid nitrogen. When leaf tissue had been thoroughly homogenized, 1ml extraction buffer (0.1M citrate phosphate buffer, pH6.5) was added (per leaf disk) and samples further homogenised with a pestle. Homogenate was poured into an Eppendorf tube, and the mortar and pestle rinsed with an additional 1ml extraction buffer which was added to the same tube. Samples were centrifuged at 12 000rpm for 10min at 4°C , and protein content of supernatant determined.

In general, protein content of extracts was determined according to the method described by Bradford (1976). Plant extracts ($5\mu\text{l}$) were diluted with water to a volume of $800\mu\text{l}$ before the addition of Bradford colour reagent ($200\mu\text{l}$; Bio-Rad, UK) to give a final volume of 1ml. The reaction solution was incubated at room temperature for 30 min, after which the absorbance of the solution was determined on a spectrophotometer at a wavelength of 595nm. Values were compared to a bovine serum albumin (BSA) standard consisting of 0, 1, 2, 5, 10, 15, or $20\mu\text{g}$ BSA in diluted Bradford colour reagent (20%, v/v; Bio-Rad, UK), which was also incubated and measured as described above. All measurements were done in duplicate.

ii) Chlorophyll

For the leaf chlorophyll profile, a disk (9.62cm^2) was cut at a distance approximately 13.5-24.5cm from the leaf tip. Disks were frozen in liquid nitrogen and stored at -80°C until chlorophyll content was determined.

Chlorophyll content was determined according to the method of Lichtenthaler and Wellburn (1983). Leaf tissue was first ground in an ice-cold mortar with a pestle, using liquid nitrogen. Each leaf disk was extracted in 1ml ice-cold acetone (80%). Homogenate was decanted into an Eppendorf tube, and mortar and pestle rinsed with an additional 1ml ice-cold acetone, which was added to the same tube. All samples were incubated at -20°C over night in the dark for complete chlorophyll extraction, before centrifuging at

14500rpm for 5min at room temperature. Samples (50 μ l) were diluted with acetone (80%) before absorbance was measured using a quartz cuvette in a spectrophotometer at wavelengths of 645nm and 663nm. Chlorophyll (mg l⁻¹) was calculated using the equation: chlorophyll (mg l⁻¹) = 20.2 x A₆₄₅ + 8.02 x A₆₆₃.

3.3.5 Statistical methods

The analysis of variance between mean values was compared using the Duncan multiple range test at P < 0.05.

3.4 Results

3.4.1 Effects of CO₂ enrichment on epidermal cell structure and stomatal densities on adaxial and abaxial leaf surfaces

Maize plants grown with CO₂ enrichment were significantly taller (23%; Fig. 3.4) than those grown at 350 μ l l⁻¹ CO₂ although they had the same number of leaves (Table 3.1). The plants grown at 700 μ l l⁻¹ had similar numbers of tillers and cobs to those grown at 350 μ l l⁻¹ (Table 3.1).



Fig. 3.4 The effect of doubling the concentration of atmospheric CO₂ from 350 μ l l⁻¹ to 700 μ l l⁻¹ on maize plants.

Table 3.1 The effect of growth CO₂ on the growth of maize plants. Plants were grown for 2 months at either 350µl l⁻¹ CO₂ or 700µl l⁻¹ CO₂. Each data point represents the mean ± SD of 13 plants per treatment, with an average of three experiments. The different letters represent statistical differences at P < 0.05.

CO ₂ (µl l ⁻¹)	Height (cm)	Number of		
		Leaves	Cobs	Tillers
350	122 ± 6 ^b	13 ± 1 ^a	2 ± 0 ^a	0 ± 0 ^a
700	150 ± 8 ^a	13 ± 1 ^a	2 ± 0 ^a	1 ± 1 ^a

The epidermal cells were arranged in parallel rows with stomata in every third or fourth row (Fig. 3.5). This pattern was similar on the adaxial (Fig. 3.5 A and C) and abaxial (Fig. 3.5 B and D) surfaces of plants grown either at 350µl l⁻¹ or 700µl l⁻¹ CO₂. However, the epidermal cells on both adaxial and abaxial surfaces were larger in the plants grown at 700µl l⁻¹ (Fig. 3.5 C and D) than in those grown at 350µl l⁻¹ CO₂. (Fig. 3.5 A and B).

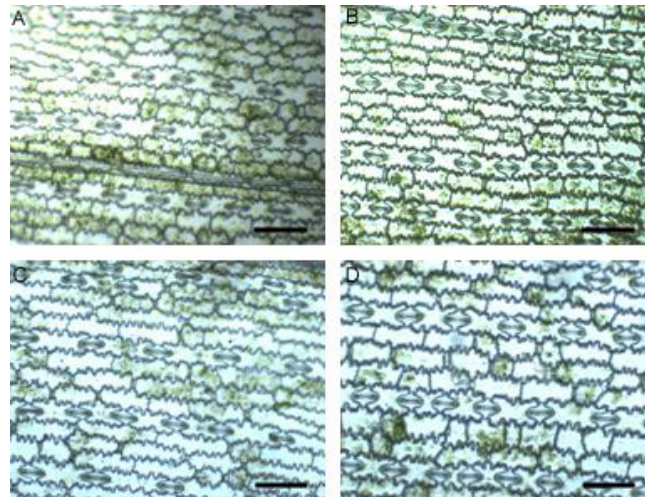


Figure 3.5 The effect of growth CO₂ on maize leaf epidermal structure. The light micrographs are representative of the structures of the adaxial surfaces of the fifth leaves of plants grown at either 350µl l⁻¹ CO₂ (A) or 700µl l⁻¹ CO₂ (C) and the abaxial surfaces of the leaves grown at 350µl l⁻¹ CO₂ (B) or 700µl l⁻¹ CO₂ (D). The bar scale is: 100µm.

The average epidermal cell area was significantly greater in 700µl l⁻¹ CO₂ -grown leaves than those grown at 350µl l⁻¹ CO₂ (Table 3.2). The smallest epidermal cells were observed on the abaxial surface of leaves grown at 350µl l⁻¹ CO₂ while the largest were on the adaxial surface of leaves grown at 700µl l⁻¹ CO₂ (Table 3.2). In contrast to epidermal cell area, which increased by about 40%, epidermal cell numbers decreased by about 30% in leaves grown at 700µl l⁻¹ CO₂. While the number of stomata was unaffected by CO₂ concentration, the size of the stomata was increased by growth at 700µl l⁻¹ CO₂ compared with 350µl l⁻¹ CO₂. The stomatal index increased as a result of doubling the CO₂

concentration on both leaf surfaces (Table 3.2). The area occupied by stomata was greater on the abaxial surface than the adaxial surface of the leaves under both growth CO₂ conditions. However, while doubling CO₂ concentration increased this parameter by over 30% on the adaxial surface, the effect was much less pronounced on the abaxial side of the leaf where stomatal area was increased by less than 20%.

Table 3.2 The structure of the adaxial and abaxial epidermis of maize leaves grown to maturity in either low (350µl l⁻¹) or high (700µl l⁻¹) CO₂.

	350µl l ⁻¹ CO ₂		700µl l ⁻¹ CO ₂	
	Adaxial	Abaxial	Adaxial	Abaxial
Epidermal cell area (µm ²)	2501 ± 341 ^c	2140 ± 285 ^d	3488 ± 504 ^a	3066 ± 615 ^b
Epidermal cells (number mm ⁻²)	467 ± 23 ^a	493 ± 15 ^a	315 ± 36 ^b	341 ± 33 ^b
Stomatal area (µm ²)	753 ± 146 ^c	918 ± 69 ^b	1006 ± 81 ^a	1072 ± 139 ^a
Stomata (number mm ⁻²)	71 ± 15 ^b	100 ± 19 ^a	67 ± 15 ^b	97 ± 15 ^a
Stomatal index	13.2 ^c	16.9 ^b	17.5 ^b	22.1 ^a
Ratio of stomata (adaxial/abaxial)	0.71		0.69	

Data represents the average ± SD for three different leaves per experiment. The different letters represent statistical differences at P < 0.05.

To explore acclimation of leaf structure to CO₂ concentration further, a second series of experiments was performed where all plants were grown at 350µl l⁻¹ CO₂ until a point where leaf 5 had emerged from the leaf sheath. Leaf 5 was then marked at the leaf base to indicate the amount of the leaf lamina that had developed and emerged into low CO₂ at this point. Half of the plants were then transferred to an environment containing 700µl l⁻¹ CO₂ and all plants were then grown for a further 6 weeks until all plants had reached maturity and leaf 5 had doubled in size with sections that emerged either into either 350µl l⁻¹ or 700µl l⁻¹ CO₂ as illustrated in Fig. 3.6.

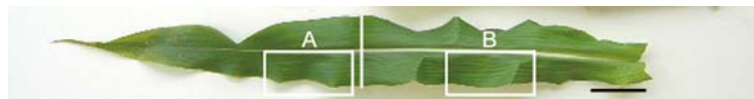


Figure 3.6. Acclimation of the 5th leaf to CO₂ enrichment. Maize plants were transferred from 350µl l⁻¹ CO₂ to 700µl l⁻¹ CO₂ at a point where half the leaf had developed and emerged into low CO₂ and plants were then grown with CO₂ enrichment to maturity. The epidermis was sampled from two different areas: one that had developed and emerged into low CO₂ and was then allowed to acclimate to high CO₂ (A) and one that had developed for 2.5 weeks at low CO₂ and thereafter (6 weeks) at 700µl l⁻¹ CO₂ (B). The scale bar is 4cm.



At this point, the section of the leaf that had emerged in air (Fig. 3A) had fewer larger epidermal cells (Table 3.3) than the part of the leaf that had emerged into high CO₂ (Fig. 3.6 B). However, the epidermal cells were large on both parts of the leaf, resembling those present on high CO₂-grown leaves rather than those grown at 350µl l⁻¹ CO₂ alone. In particular, while the epidermal cells in the part of the leaf that had emerged into 700 µl l⁻¹ CO₂, were similar in both types of experiments (compare the data for 700µl l⁻¹ in Tables 3.2 and 3.3) the cells on the parts of the leaves that had emerged into air and were then transferred to 700µl l⁻¹ CO₂ tended to be even larger than those that had emerged from the leaf sheath directly into 700µl l⁻¹ CO₂ (Table 3.2).

Table 3.3 A comparison of acclimation effects on the structure of the adaxial and abaxial epidermis. Samples from the 5th maize leaf that had emerged and grown at 350µl l⁻¹ CO₂ for 2.5 weeks and thereafter at 700µl l⁻¹ CO₂ (A) are compared with sections that had emerged and grown at 700µl l⁻¹ CO₂ (B). Data represents average ± SD for three different leaves per experiment. The different letters represent statistical differences at P < 0.05.

	(A) Emerged in 350µl l ⁻¹ CO ₂		(B) Emerged in 700µl l ⁻¹ CO ₂	
	Adaxial	Abaxial	Adaxial	Abaxial
Epidermal cell area (µm ²)	3755 ± 430 ^a	3842 ± 589 ^a	3169 ± 772 ^b	2913 ± 458 ^b
Epidermal cells (number µm ⁻²)	234 ± 33 ^b	269 ± 77 ^{ab}	284 ± 71 ^{ab}	322 ± 60 ^a
Stomata (number µm ⁻²)	57 ± 10 ^b	82 ± 16 ^a	60 ± 13 ^b	82 ± 15 ^a
Ratio of stomata (adaxial/abaxial)	0.7		0.73	

While the adaxial surface always had fewer stomata than the abaxial surface regardless of growth CO₂ concentration, there was no statistically significant difference in epidermal cell area, number of epidermal cells per µm², number of stomata per µm² or stomatal ratio of either the adaxial or abaxial epidermis between parts of the leaf grown at 350µl l⁻¹ CO₂ for 2.5 weeks and subsequently transferred to 700µl l⁻¹ CO₂ and those that had only been exposed to 700µl l⁻¹ CO₂ (Table 3.3). It is concluded that the section of leaf that had emerged into air had fully acclimated to higher CO₂ concentration in terms of structure during the 6 weeks growth at 700µl l⁻¹ CO₂.

3.4.2 Acclimation of leaf chlorophyll and protein to CO₂ enrichment

Chlorophyll (Fig. 3.7 A) and protein (Fig. 3.7 B) contents were determined in leaves of mature plants grown either at 350µl l⁻¹ CO₂ or 700µl l⁻¹ CO₂ and in those grown at 350µl l⁻¹

^{14}C CO₂ for 2.5 weeks (to the leaf 5 stage) and transferred to an environment containing 700 $\mu\text{l l}^{-1}$ CO₂ (Fig. 3.7).

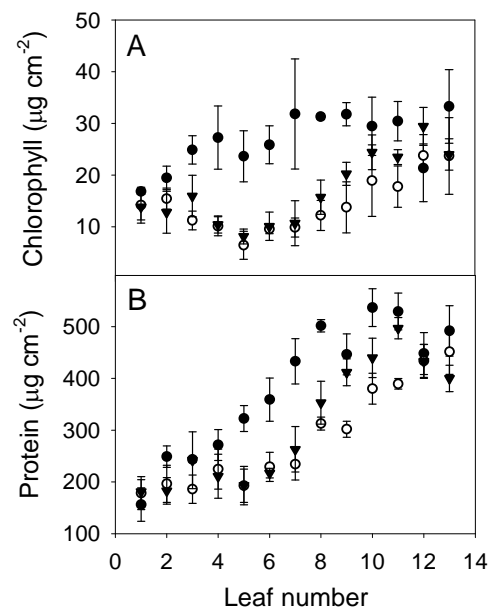


Figure 3.7 The effect of CO₂ enrichment on the chlorophyll (A) and protein (B) content of the leaves on fully mature maize plants (as shown in Fig. 3.4). Measurements were made from the lowest leaf (1) to uppermost mature leaf (13) in plants grown at either 350 $\mu\text{l l}^{-1}$ CO₂ (filled circles), 700 $\mu\text{l l}^{-1}$ CO₂ (open circles), or at 350 $\mu\text{l l}^{-1}$ CO₂ for 2.5 weeks and thereafter at 700 $\mu\text{l l}^{-1}$ CO₂ (inverted triangles). The complete experiment involving 12-14 plants was repeated three times. Data show the mean values \pm SE in each case (n=3).

The youngest (leaves 12 and 13) had similar amounts of leaf chlorophyll and protein regardless of growth CO₂ as did the oldest leaves (leaves 1 and 2). All other leaves on the plants, particularly the middle leaves, had markedly lower chlorophyll and protein at 700 $\mu\text{l l}^{-1}$ CO₂ compared with 350 $\mu\text{l l}^{-1}$ CO₂. Similarly, leaves of plants transferred after 2.5 weeks from 350 $\mu\text{l l}^{-1}$ CO₂ to 700 $\mu\text{l l}^{-1}$ CO₂ showed lower levels of chlorophyll and leaf protein comparable to values measured in plants that had continuously experienced only 700 $\mu\text{l l}^{-1}$ CO₂ (Fig. 3.7). These data support the conclusion that the leaves that had originally emerged into air had fully acclimated to higher CO₂ concentration during the 6 weeks growth at 700 $\mu\text{l l}^{-1}$ CO₂.

3.4.3 Photosynthesis rates in mature source leaves

As shown in Fig. 3.7, mature source leaves of plants grown at 350 $\mu\text{l l}^{-1}$ CO₂ had a total chlorophyll content of $26.71 \pm 5.24 \mu\text{g cm}^{-2}$ and a protein content of $383.12 \pm 124.64 \mu\text{g cm}^{-2}$, whereas those from plants grown at 700 $\mu\text{l l}^{-1}$ CO₂ had a total chlorophyll content of

$14.38 \pm 5.39 \mu\text{g cm}^{-2}$ and a protein content of $286.71 \pm 97.30 \mu\text{g cm}^{-2}$. Similarly, plants that were grown at $350 \mu\text{l l}^{-1} \text{CO}_2$ 2.5 weeks, and subsequently transferred to $700 \mu\text{l l}^{-1} \text{CO}_2$ had a total chlorophyll content of $16.9 \pm 6.8 \mu\text{g cm}^{-2}$ and a protein content of $312.2 \pm 113.1 \mu\text{g cm}^{-2}$.

While the whole leaves of plants grown at $700 \mu\text{l l}^{-1} \text{CO}_2$ had lower photosynthesis rates on a surface area basis compared with plants that were grown at $350 \mu\text{l l}^{-1} \text{CO}_2$ they had higher photosynthesis rates on a chlorophyll basis (Fig. 3.8; Table 3.4). The average rate of CO_2 assimilation for whole leaves, measured at $350 \mu\text{l l}^{-1} \text{CO}_2$, in plants grown at $350 \mu\text{l l}^{-1} \text{CO}_2$ was $394 \pm 168 \mu\text{mol h}^{-1} \text{mg}^{-1} \text{chl}$ whereas the rate in leaves grown at $700 \mu\text{l l}^{-1} \text{CO}_2$ at $350 \mu\text{l l}^{-1} \text{CO}_2$ was $810 \pm 258 \mu\text{mol h}^{-1} \text{mg}^{-1} \text{chl}$.

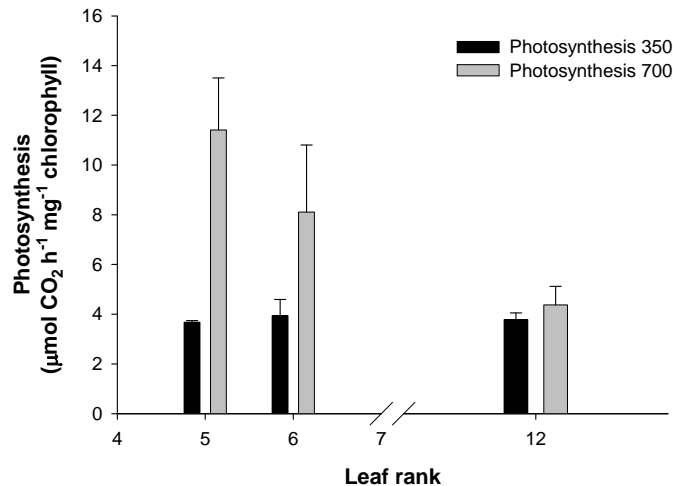


Figure 3.8 Photosynthesis rates measured in leaves 5, 6, and 12 of mature maize plants grown in air ($350 \mu\text{l l}^{-1} \text{CO}_2$, “350”) or with CO_2 enrichment ($700 \mu\text{l l}^{-1} \text{CO}_2$, “700”).

Table 3.4 Comparison of photosynthesis rates in leaves grown in air or with CO_2 enrichment, based on leaf surface area or chlorophyll (Chl) content.

Growth CO_2 ($\mu\text{l l}^{-1}$)	Leaf rank	Photosynthesis measured on	Total leaf chlorophyll content (mg m^{-2})	Photosynthesis (per area) ($\mu\text{mol m}^{-2} \text{h}^{-1}$)	Photosynthesis (per Chl) ($\mu\text{mol mg}^{-1} \text{h}^{-1}$)
350	12	whole leaf	332.72	1257.60	3.78
350	6	abaxial side	258.50	1020.00	3.95
350	5	whole leaf	236.31	868.80	3.68
700	12	whole leaf	236.96	1036.80	4.38
700	6	abaxial side	95.48	774.00	8.11
700	5	whole leaf	64.33	734.40	11.42

3.4.4 CO₂ response curves for photosynthesis on the adaxial and abaxial leaf surfaces

Increasing ambient CO₂ (C_a) caused different responses in gas exchange on the adaxial and abaxial surfaces of maize leaves. This effect was examined in two maize hybrids: H99 (Fig. 3.9) and Hudson (Fig. 3.10), which had different absolute photosynthetic capacities, rates in Hudson being about twice those measured in H99. These hybrids were grown to maturity at either 350 μL l⁻¹ CO₂ or 700 μL l⁻¹ CO₂. Photosynthetic CO₂ assimilation rates were consistently higher on the abaxial surfaces than the adaxial surfaces in both H99 (Fig. 3.9 A and B) and Hudson (Fig. 3.10 A and B), regardless of the growth CO₂. Photosynthetic rates on both surfaces were lower in plants grown at 700 μL l⁻¹ CO₂. However, the kinetics of the CO₂ response curve for photosynthesis was very different on the two leaf surfaces. On the abaxial surface, photosynthesis increases with CO₂ concentration until maximal assimilation rates are reached and rates thereafter remain stable as ambient CO₂ concentration is increased. This is not the case on the adaxial surface where maximal assimilation rates are much lower than on the abaxial surface. Moreover, while maximal photosynthetic rates are attained at about the ambient CO₂ concentration sat which plants had been grown, higher CO₂ concentrations inhibited photosynthesis.

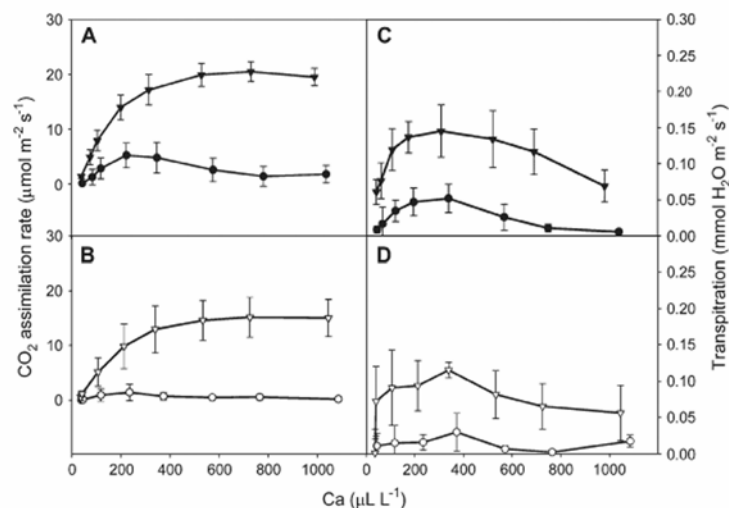


Figure 3.9 CO₂ response curves for photosynthesis (A, B) and transpiration rates (C and D) on the adaxial (filled/open circles) and abaxial (filled/open inverted triangles) surface of *Zea mays* hybrid H99 leaves. Plants were grown at either 350 μL l⁻¹ CO₂ (A and C) or 700 μL l⁻¹ CO₂ (B and D). The experiment was repeated three times with leaves from six plants measured in each experiment. Data show the mean values ± SE in each case (n=3).

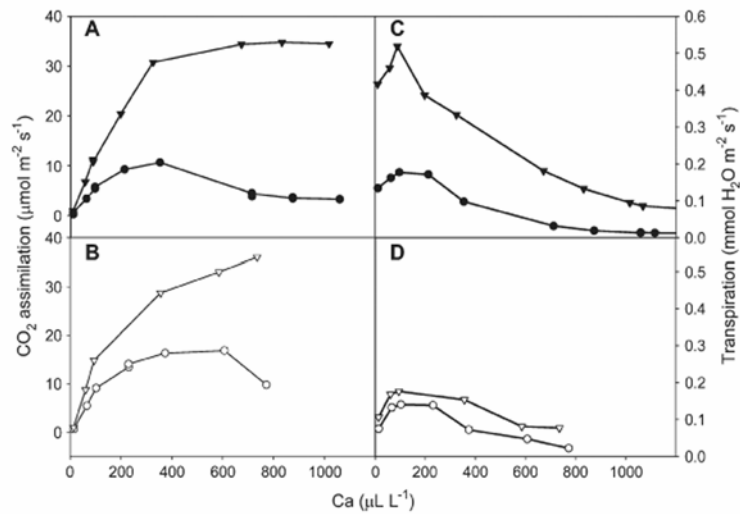


Figure 3.10 CO₂ response curves for photosynthesis (A and B) and transpiration rates (C and D) on the adaxial (filled/open circles) and abaxial (filled/open inverted triangles) surface of *Zea mays* hybrid Hudson leaves. Plants were grown at either 350 μl l⁻¹ CO₂ (A and C) or 700 μl l⁻¹ CO₂ (B and D). The complete experiment was repeated three times but the figure shows a single representative curve in each case.

The stomatal index was considerably increased in plants grown at 700 μl l⁻¹ CO₂. The stomatal index was lowest on the adaxial (Fig. 3.11, i) surfaces of maize leaves grown at 350 μl l⁻¹ CO₂. Calculated values were higher on the abaxial (Fig. 3.11, ii) surfaces in both growth conditions. Increasing the growth CO₂ concentration affected the relationship between stomatal index and CO₂ assimilation rate (Fig. 3.11 A) in a similar manner to that observed with regard to transpiration rates (Fig. 3.11 B).

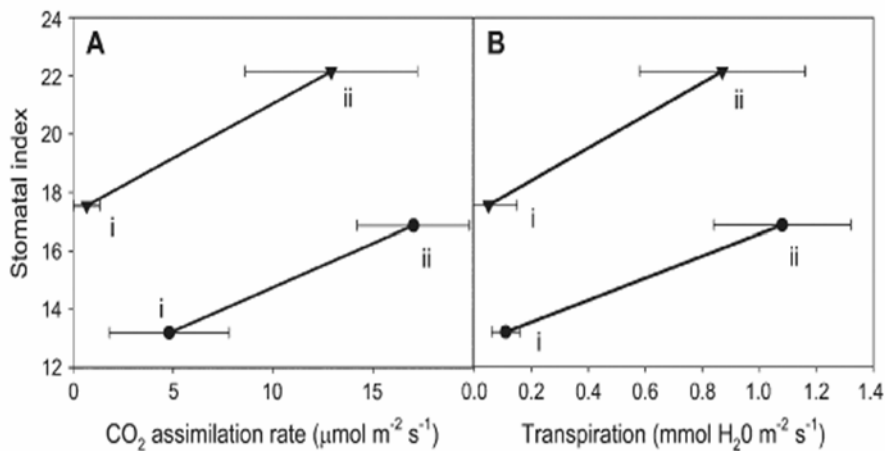


Figure 3.11 Relationships between photosynthesis, transpiration rates and stomatal index on the adaxial (i) and the abaxial (ii) surfaces of maize leaves grown either at 350 μl l⁻¹ CO₂ (closed circles) or 700 μl l⁻¹ CO₂ (filled inverted triangles).

3.4.5 The effect of light orientation on photosynthetic CO₂ responses

In the above experiments irradiance had been supplied only on the upper adaxial surface. To test whether the direction of the irradiance had a direct effect on CO₂ uptake from each surface, CO₂ assimilation was measured with irradiance supplied first to the adaxial side of the leaf until a steady rate of photosynthesis was observed, and then with irradiance supplied to the abaxial side of the leaf.

Steady-state CO₂ assimilation rates were established by incubating the leaves for 30 min at 780 μl l⁻¹ CO₂ with light on the adaxial surface (Fig. 3.12 i). The leaves were then inverted so that the light entered the leaves via the abaxial surfaces (Fig. 3.12 ii). The low CO₂ uptake rates observed on the adaxial surfaces in the standard orientation did not recover once the leaves were inverted and light was applied directly to the abaxial surfaces (Fig. 3.12 i). Moreover, while leaf inversion caused an initial transient decrease in the photosynthesis rate on the abaxial surface this rapidly recovered (Fig. 3.12 ii). Even after several h in this condition no subsequent changes in CO₂ uptake rates were observed.

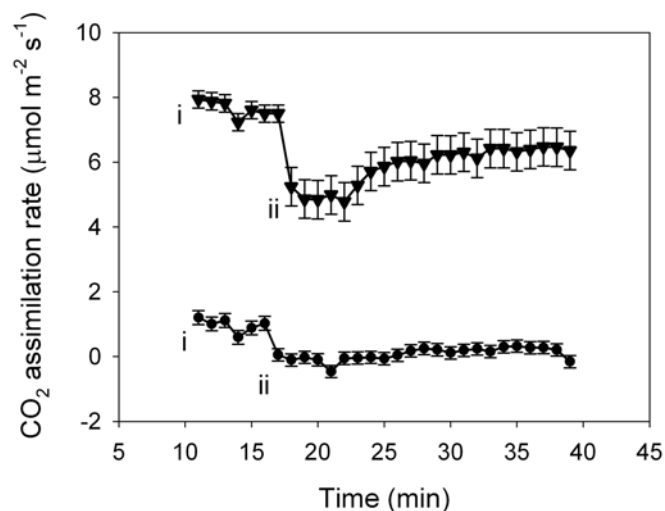


Figure 3.12 The effect of irradiance on CO₂ assimilation rates when applied directly either to the adaxial or the abaxial leaf surfaces. The light input was orientated first from the adaxial side (i) until steady-state rates of photosynthesis had been attained (30 min after the onset of illumination). Then the light source was switched to the abaxial surface (ii). CO₂ assimilation rates on the adaxial surface (filled circles) and abaxial surface (filled inverted triangles) were measured simultaneously at 780 μl l⁻¹ CO₂ in plants grown at 780 μl l⁻¹ CO₂. The experiment was repeated three times with leaves from six plants measured in each experiment. Data show the mean values ± SE in each case.



3.5 Discussion

The developmental and physiological consequences of elevated CO₂ concentration on leaf structure and function are of particular relevance in maize as it is a major food crop. The effects of climate change occasioned by anthropogenic release of CO₂ has been considered many times in relation to crops, with C₄ plants predicted to respond only marginally to future elevated CO₂ concentrations (Poorter and Navas, 2003). The results presented here indicate that maize plants show a very significant positive response to doubling ambient growth CO₂ concentrations. While early studies on the effects of CO₂ enrichment on C₄ photosynthesis had led to the prediction that maize photosynthesis would not be enhanced by elevated atmospheric CO₂, recent work in Free-Air CO₂ enrichment (FACE) experiments has established that maize leaf photosynthesis can be increased by elevated CO₂ (Leakey et al., 2004; Long et al., 2004). Similarly, the FACE studies have provided little evidence of the photosynthetic acclimation observed in C₄ species in controlled environment chamber and glasshouse studies (Long et al., 2004). In the present study conducted on plants grown in controlled environment cabinets and rooms, CO₂ assimilation rates measured on a surface area basis were decreased as a result of growth at high CO₂, but the plants were much taller as a consequence of CO₂ enrichment.

Mature leaves detect and regulate the CO₂ response of stomatal initiation in developing leaves (Lake et al., 2001 and 2002) with stomatal densities decreasing by about 20–30% for a doubling of atmospheric CO₂ (Woodward, 2002). Moreover, CO₂ signalling from mature leaves determines the photosynthetic potential of the developing leaves (Lake et al., 2002; Woodward, 2002). The presence of stomata on the upper adaxial surface increases maximum leaf conductance to CO₂ and the overall value of varying stomatal densities on the upper and lower leaf surfaces has been discussed in terms of decreasing diffusion limitations to photosynthesis in thick leaves with high photosynthetic capacities (Mott et al., 1982; Mott and Michaelson, 1991). Gas exchange characteristics from the surfaces of amphistomatous leaves have previously been described in detail particularly in relation to light intensity (Mott and O’Leary, 1984; Mott and Michaelson, 1991; Mott et al., 1982, 1993; Anderson et al., 2001) but little information is available on responses to CO₂ enrichment. Stomata on the upper and lower surfaces on amphistomatous leaves respond differently to environmental factors, but this response is not an adaptation to



different CO₂ exchange characteristics on the two surfaces (Mott and O'Leary, 1984). The results presented here allow the following conclusions to be drawn.

CO₂ enrichment modifies epidermal cell expansion in maize leaves

The adaxial surface of the maize leaves always had fewer stomata than the abaxial surface regardless of growth CO₂ concentration. However, increasing the atmospheric CO₂ resulted in fewer, larger epidermal cells in which a similar number of larger stomata are interspersed. Thus, the stomatal index increased as a result of CO₂ enrichment. The transfer experiments from low to high CO₂ confirmed that the maize leaf epidermal cells rapidly acclimate to CO₂ enrichment. Six weeks after transfer to 700 μl l⁻¹ CO₂ the epidermal cells on both parts of the leaf were large and resembled those present on high CO₂-grown leaves rather than those grown at 350 μl l⁻¹ CO₂. Moreover, the cells on the parts of the leaves that had developed and emerged into air and then been transferred to 700 μl l⁻¹ CO₂ were even larger than those that had developed and emerged from the leaf sheath directly into 700 μl l⁻¹ CO₂. This would suggest that stimulation of cell expansion and cell enlargement is a primary acclimatory response to CO₂ enrichment.

There was no statistically significant difference in the number of stomata per unit surface (mm²) or stomatal/epidermal cell ratio in parts of the leaf that had emerged into 350 μl l⁻¹ CO₂ and had then been transferred to high CO₂ and those that had emerged into high CO₂ alone. These data confirm that, unlike epidermal cell area that shows an acclimatory response to prevailing CO₂, stomatal patterns are fixed prior to emergence. In Arabidopsis, local high CO₂ in the developing leaf environment negated the signal for increased density arising from mature leaves maintained at a low CO₂ (Lake et al., 2002). In maize, the epidermal cell numbers and epidermal and stomatal cell sizes are highly responsive to environmental CO₂ concentration. Hence, changes in epidermal cell numbers are largely responsible for CO₂-induced increases in stomatal index rather than a CO₂ effect on stomatal numbers *per se*.

CO₂ enrichment causes acclimation of maize leaf photosynthesis

Maize, like sorghum (Watling et al., 2000), showed extensive acclimation to growth at high CO₂. While plants with higher stomatal densities generally have high stomatal conductance and photosynthetic rates (Lake et al., 2002), the stomatal area measured here in the 700 μl l⁻¹ CO₂-grown maize was slightly higher than that of plants grown at 350 μl l⁻¹



CO₂ despite similar rates of photosynthesis. However, acclimation of leaf chlorophyll and protein was evident in plants grown at 700 μl l⁻¹ CO₂, which had much lower levels of both parameters than plants grown at 350 μl l⁻¹ CO₂. On average, plants grown at 350 μl l⁻¹ CO₂ had 58% more chlorophyll and 29% more protein than plants grown at 700 μl l⁻¹ CO₂. Lower levels of chlorophyll and leaf protein were also observed in plants transferred to 700 μl l⁻¹ CO₂ after 2.5 weeks growth at 350 μl l⁻¹ CO₂. Hence, all leaves rapidly acclimated to CO₂ enrichment after transfer. While growth at high CO₂ led to a slight decrease in leaf photosynthesis on a surface area basis, acclimation was associated with a large increase in the efficiency of photosynthesis, which was doubled on a chlorophyll basis in plants grown at the higher CO₂ concentration. These results show that maize leaves acclimate well to growth at high CO₂ and benefit from CO₂ enrichment.

CO₂ enrichment has a different effect on photosynthesis of the adaxial and abaxial leaf surfaces

The amphistomatous nature of maize leaves means that they have the capacity to open and close their stomata on both sides independently, with transpiration rates being more sensitive to changes in stomatal aperture on the abaxial surface. The data presented here shows that atmospheric CO₂ has a pronounced differential effect on photosynthetic CO₂ uptake rates on the adaxial and abaxial leaf surfaces. The CO₂ response curves for photosynthesis on the adaxial and abaxial surfaces showed that in both cases uptake increased with increasing CO₂ concentration up to growth CO₂. Above these values, CO₂ assimilation rates showed different responses to ambient CO₂ on the adaxial and abaxial surfaces. While assimilation on the abaxial surface was stable or increased as CO₂ concentration increased, assimilation on the adaxial surface decreased as CO₂ concentration increased. The physiological significance of the observed stomatal polarity with regard to the regulation of CO₂ uptake rates is unknown and it is surprising given that the C₄ maize leaf is essentially non-polar with similar distances for light and CO₂ to travel on either side of the leaf. Further work is required to determine the structural mechanisms contributing to this marked functional polarity, for example, perhaps the chloroplasts are arranged somewhat differently in the photosynthetic cells beneath the adaxial and abaxial epidermal surfaces. However, it may be that this phenomenon is a specific feature of C₄ leaves as such differential controls of photosynthesis have been observed on the adaxial and abaxial surfaces of another C₄ species, *Paspalum*, but not in the leaves of the C₃ species, wheat (Soares et al., 2008).



The decrease in photosynthesis on the adaxial leaf surfaces in response to high CO₂ is not necessarily related to water use efficiency

The differential photosynthetic assimilation rates observed on the adaxial and abaxial surfaces in response to ambient CO₂ were not influenced by the direction of light entry to the leaf. Improved plant water status is considered to be the primary basis for higher CO₂ assimilation rates in C₄ plants under elevated CO₂. However, the results presented here suggest that the function of stomata with regard to control of photosynthesis is genetically programmed to be different on the leaf surfaces. Given that transpiration rates are more sensitive to changes in stomatal aperture on the abaxial surface, it is surprising that this population has a much higher threshold for closure in response to high CO₂ and high light than the adaxial surface population. This has important implications for the control of plant water loss even though the maize plants studied here were well watered. These results would argue against a simple stomata-based strategy for optimizing water-use efficiency in maize and indicate that the stomata on the upper leaf surface are programmed to be a much more sensitive barometer for changes in ambient CO₂ than those on the lower surface, which remain open even at excessively high CO₂ levels.

Overall, this part of the study has shown that maize acclimates substantially to an increased availability of CO₂ by decreasing protein and chlorophyll content, and increasing stomatal index. In order to identify changes in the maize transcriptome and possible signalling molecules that might underpin these acclamatory responses, a microarray study was undertaken which is discussed in the following chapter.