Acclimation to high CO_2 in maize is dependent on leaf rank.

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Abstract

The responses of C_3 plants to rising atmospheric CO_2 levels are considered to be largely dependent on effects exerted through altered photosynthesis. In contrast, the nature of the responses of C_4 plants to high CO_2 remains controversial because of the absence of CO_2-dependent effects on photosynthesis. In this study, the effects of atmospheric CO_2 availability on the transcriptome, proteome and metabolome profiles of two ranks of source leaves in maize (*Zea mays* L.) were studied in plants grown under ambient CO_2 conditions (350+/- 20 μL L^{-1} CO_2) or with CO_2 enrichment (700+/- 20 μL L^{-1} CO_2). Growth at high CO_2 had no effect on photosynthesis, photorespiration, leaf C/N ratios or anthocyanin contents. However, leaf transpiration rates, carbohydrate metabolism and protein carbonyl accumulation were altered at high CO_2 in a leaf-rank specific manner. While no significant CO_2-dependent changes in the leaf transcriptome were observed, qPCR analysis revealed that the abundance of transcripts encoding a Bowman-Birk protease inhibitor and a serpin were changed by the growth CO_2 level in a leaf rank specific manner. Moreover, CO_2-dependent changes in the leaf proteome were most evident in the oldest source leaves. Small changes in water status may be responsible for the observed responses to high CO_2, particularly in the older leaf ranks.

Keywords: CO_2 enrichment, protease inhibitors, sugar signalling, redox regulation, CO_2 assimilation

Introduction

Global atmospheric CO_2 concentrations have risen from about 270 μL. L^{-1} in pre-industrial times to the present level of over 380 μL. L^{-1}. Values are predicted to reach between 530 and 970 μL. L^{-1} by the end of this century. Moreover, the capacity of the earth's oceans to absorb CO_2 from the atmosphere is considered to be reaching saturation point (Khatiwala *et al*. 2009). Average global temperatures have increased by about 0.76°C over the last 150 years and they are likely to increase by another 1.7° to 3.9°C during this century. In theory, plants could mitigate these changes through photosynthetic conversion of atmospheric CO_2 into carbohydrates and other organic compounds. However, the potential for this mitigation remains uncertain and the impact of predicted increases in atmospheric CO_2 and global temperatures on plant productivity is a growing concern for agriculture and food security worldwide (Long *et al*. 2004; Long *et al*. 2006). In plants that use the C_3 pathway of photosynthesis, the predicted changes in atmospheric CO_2 concentrations and the temperature of the earth will directly influence the balance between photosynthetic carbon fixation and photorespiration, and also plant C/N relationships as atmospheric CO_2 enrichment inhibits the assimilation of nitrate into organic nitrogen compounds (Foyer *et al*. 2009). The anticipated changes in the Earth's environment will therefore influence partitioning between these pathways, and as a consequence influence the distribution of plants with different carbon fixation pathways (Wand *et al*. 1999; Ward *et al*. 1999; Zhu, Goldstein & Bartholomew 1999). In the C_3 pathway of photosynthesis, CO_2 enters the Benson-Calvin cycle directly by the action of ribulose 1, 5-bisphosphate carboxylase oxygenase (Rubisco). In contrast, the C_4 pathway of photosynthesis incorporates an endogenous CO_2-concentrating mechanism where CO_2 is first incorporated into organic acids in the mesophyll cells. By virtue of this CO_2-

concentrating mechanism CO_2-limitations at the Rubisco active site in the bundle sheath cells are diminished. Hence, increasing atmospheric CO_2 is predicted to have less impact on plants with the C_4 pathway of photosynthesis than their C_3 counterparts.

Elevated CO_2 atmospheric concentrations decrease photorespiration and initially enhance photosynthesis and growth as much as 35% in most C_3 plants (Long *et al*. 2004; Long *et al*. 2006). The high CO_2–dependent enhancement of photosynthesis, however, diminishes over time (days to years), a phenomenon known as CO_2 acclimation (Paul & Foyer, 2001). This process, which involves loss of Rubisco protein and activity, is largely absent from C_4 plants, where these enzymes have already acclimated to functioning under high CO_2 conditions. However, there is considerable variation in the responses of different C_4 species to enhanced CO_2 (Maroco, Edwards & Ku 1999; Ziska & Bunce 1999; Ward *et al*. 1999; Cousins *et al*. 2001). While C_4 plants show acclimation to high CO_2 (Soares *et al*. 2007) this is generally not related to effects on photosynthesis, which is largely unchanged (Sage 1994; Ghannoum *et al*. 1997 & 2001; von Caemmerer *et al*. 2001, Wand *et al*. 2001; Leakey *et al*. 2006).

Atmospheric CO_2 availability exerts a strong influence on stomatal density and patterning (Larkin *et al*. 1997; Croxdale 1998; Taylor *et al*. 1994, 2008; Masle 2000; Lake *et al*. 2001, Lake, Woodward & Quick 2002; Poorter & Navas 2003; Martin & Glover 2007). Signals concerning CO_2 availability appear to be transmitted from older to developing leaves in C_3 plants (Lake *et al*. 2001; 2002; Woodward 2002). Growth at enhanced atmospheric CO_2 concentrations often leads to a decrease in stomatal density (Woodward, Lake & Quick 2002). However, this acclimation response varies

considerably between species (Woodward & Kelly 1995). High CO_2–dependent increases in stomatal index have been observed in two monocotyledonous C_4 species, maize and *Paspalum dilatatum* (Driscoll *et al*. 2006; Soares *et al*. 2007, 2009). The CO_2-signalling pathways that orchestrate such changes in epidermal patterning and leaf structure, function and composition remain poorly characterized (Gray *et al*. 2000; Ferris *et al*. 2002) but it is generally accepted that the signals that regulate such responses are transported from mature to developing leaves (Coupe *et al*. 2006; Miyazawa *et al*. 2006). Carbonic anhydrases have recently been considered to be important components of plant CO_2-signalling pathways (Hu *et al*. 2010). *Arabidopsis thaliana* double-mutants lacking two B-carbonic anhydrases (B-CA1 and B-CA4) had impaired increased stomatal densities but impaired stomatal movements in response to changing CO_2 levels (Hu *et al*. 2010).

The stability of internal CO_2 (C_i) with changing CO_2 fixation rates suggests that leaves are able to sense and signal information concerning C_i (Warren 2008), rather than atmospheric CO_2 (Mott 1988). The guard cells of stomata are able to sense an array of endogenous and environmental signals including CO_2 availability. Atmospheric CO_2 can induce either stomatal opening or closure depending on concentration by influencing the patterns of repetitive calcium transients within the guard cells that are linked to changes in ion channel activities and potassium efflux. Like abscisic acid (ABA), high CO_2 participates in a complex network of signalling events that cause an inhibition of K^+ inward rectifying channels and an activation of K^+ outward rectifying channels that enhances potassium efflux and consequently results in stomatal closure (Acharya & Assmann, 2009).

The responses of leaf cells to C_i are not restricted to the stomata (von Caemmerer *et al*. 2004; Baroli *et al*. 2008; Mott *et al*. 2008). However, compared to the extensive literature on systemic light signalling responses (Karpinski *et al*. 1999; Rossel *et al*. 2007; Muhlenbock *et al*. 2008), little information is available concerning systemic CO_2 signalling pathways in plants (Mott, Sibbernsen & Shope 2008; Baroli *et al*. 2008) and very few CO_2 signalling components have been identified (Gray *et al*. 2000). Systemic light signalling is triggered by the photosynthetic electron transport chain in the high-light-exposed leaves and involves salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) dependent pathways (Karpinski *et al*. 1999; Fryer *et al*. 2003; Rossel *et al*. 2007; Muhlenbock *et al*. 2008). Studies concerning CO_2 signalling pathways in plants are often complicated by direct effects of CO_2 on photosynthesis, particularly in C_3 plants. To avoid this complication, we have studied how the leaves of maize, an economically important C_4 species, respond to enhanced atmospheric CO_2, availability. Maize plants were grown for up to eight weeks either under the ambient CO_2 conditions of our laboratories (350+/- 20 $\mu L\ L^{-1}$ CO_2) or with CO_2 enrichment (700+/- 20 $\mu L\ L^{-1}$ CO_2). We have combined classic whole plant physiology and -omics approaches to characterise the acclimation of two ranks of maize source leaves to high CO_2 and to use this information to provide information on possible CO_2 signalling mechanisms.

Materials and Methods

Plant material and growth conditions

Zea mays L. hybrid H99 plants were grown for up to 8 weeks in compost (Driscoll *et al*. 2006) in duplicate controlled environment cabinets (Sanyo 970, SANYO, Osaka) where the atmospheric CO_2 was maintained at either 350 μL L^{-1} or at 700 μL L^{-1}. In total six controlled environment cabinets were used in these experiments. Each cabinet contained plants grown under one of the above conditions, i.e. either 'ambient' conditions or 'high CO_2'. In total, three cabinets were set at the ambient CO_2 levels while the other three were maintained at the high CO_2 level. The data or harvests obtained from plants in each cabinet were pooled so that there is a one-to-one match of cabinets and pools.

In all cases, the plants were grown with 16 h photoperiod (700 μmol m^{-2} s^{-1}) and the temperature was maintained at 25ºC (day) and 19ºC (night) with 80% relative humidity. The CO_2 was supplied from a bulk container, transmitted via a Vaisala GMT220 CO_2 transmitter (VAISALA OYJ, Helsinki, Finland), and maintained by a Eurotherm 2704 controller (EUROTHERM LTD Worthing, U.K.) which kept CO_2 levels at 350 ± 20 μL L^{-1} or 700 ± 20 μL L^{-1}. All plants were watered daily throughout development in order to avoid a water stress. Leaf samples were harvested 11 h into the photoperiod from plants at three (6 leaves), six (9 leaves) and eight (13 leaves) weeks of growth.

Leaf parameters, water use efficiencies and photosynthesis measurements

The whole leaf weight and leaf area measurements were performed on each leaf rank at the 13-leaf stage. Leaf area measurements were performed using a ΔT area meter

(Delta-T Devices LTD, England). Intrinsic water use efficiencies were measured according to Soares-Cordeiro *et al* (2009). Photosynthetic gas exchange measurements were performed as described previously (Driscoll *et al*. 2006).

Anthocyanin, chlorophyll and pheophytin determinations

Fresh weight and dry weight measurements were performed on each leaf rank at 13-leaf stage. Leaf discs ($8cm^2$) were cut from the centre of each leaf. Leaf mass was measured before and after the tissue was dried in an oven for 3 days at 60°C. Leaf anthocyanins were extracted and assayed in separate maize discs harvested as above, according to Sims and Gamon (2002). Chlorophyll was measured in the same tissues according to Wintermans and De Mots (1965) and the pheophytin content was determined according to Vernon (1960).

Metabolome and amino acid analyses

Samples from leaf rank 5 on 9 week-old plants were harvested and frozen immediately in liquid nitrogen. Four independent extracts per plant were analyzed by GC-TOF-MS, using a method adapted from Noctor *et al*. (2007). Ninety four derivatives corresponding to 88 unique metabolites were identified by reference to their mass spectra. Peak intensities were normalized to an internal standard (ribitol) and then to leaf chlorophyll contents, and metabolites showing a statistically significant difference between the two conditions were identified by t-test ($p < 0.05$).

Leaf temperature and total C and N contents

Leaf temperature was measured on all leaf ranks of 3 week-old plants. Whole leaves were then harvested and frozen immediately in liquid nitrogen. Total C and N

contents were measured by elemental analysis (EA1108, Series 1, Carlo Erba Istrumentazione, Milan, Italy).

Measurements of leaf sucrose, hexoses and starch

Leaf sucrose, hexoses and starch measurements were performed on each leaf rank at 13-leaf stage. Whole leaves were harvested and immediately frozen in liquid nitrogen in the growth cabinets. Leaf sucrose and hexose were extracted and assayed according to Jones, Outlaw and Lowry (1977). Starch was extracted and assayed in the same samples according to the method of Paul and Stitt (1993).

Measurement of the extent of leaf protein carbonylation

Protein carbonylation assays were performed on each leaf rank at 13-leaf stage. Whole leaves were harvested and immediately frozen in liquid nitrogen in the growth cabinets. The composition and extent of protein carbonyl group formation was measured using the OxyBlot™ Oxidized Protein Detection Kit (Chemicon International, UK).

RNA extraction methods

Micro-analyses were performed on samples (in triplicate) of rank 3 and rank 12 leaves from separate plants at 13-leaf stage. Three separate sets of leaf samples were prepared per leaf rank and per treatment. Each individual sample was made up of three whole pooled leaves taken from 3 separate plants. The samples were as follows: (a) leaf rank 3 under ambient CO_2 conditions (b) leaf rank 12 under ambient CO_2 conditions, (c) leaf rank 3 under high CO_2 conditions and (d) leaf rank 12 under high CO_2 conditions. Total RNA was extracted using Trizol reagent (Invitrogen, UK).

For qPCR, RNA was extracted from pooled samples of three biological replicates grown in either air or with CO_2 enrichment, representing each leaf on the stem. For the feeding study, total RNA was extracted from 400mg of frozen tissue obtained from pooled tissue of 10 biological replicates. Total RNA was purified with RNeasy Mini Spin Columns (Qiagen).

Microarray hybridization techniques

Purified RNA samples were sent to ArosAB, Denmark, where they were converted to cDNA and used to synthesise biotin-labelled cRNA (BioArray High Yield RNA Transcript Labeling Kit, Enzo). Labelled cRNA was hybridised to maize microarray chips (Affymetrix) for samples isolated from air-grown plants giving rise to 12 microarray chips representing three biological replicates of the young and old plants grown in air and high CO_2 levels. The corn microarrays cover approximately 14k probesets, which represent less than 10k genes. Each chip was washed and scanned in a GeneChip Scanner 3000 (Affymetrix). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-1222.

Microarray analysis

Gene expression profiles were then compared using GeneSpring GX 11.00. The p-values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg FDR). A cut-off with p-value <0.05 and log2 expression ratio ±1 was adopted. The probe targets were as defined by Affymetrix (https://www.affymetrix.com/analysis/netaffx/index.affx) and these targets were used to guide annotation of the genes through the use of BLAST against the TAIR

(http://www.arabidopsis.org/) database. Transcripts were identified according to Unigene cluster (http://www.ncbi.nlm.nih.gov/UniGene; Pontius, Wagner & Schuler 2003) and translated homology search (tblastx) on the NCBI protein refseq database and on the trembl (EBI) data set with the criteria of minimum score of 50% and e-value of$\leq$10 -7 for selecting potentially significant homology to the Affymetrix supplied target sequences as the subjects for comparison.

Sugar and pro-oxidant treatments.

Ten leaves from plants grown in ambient CO_2 were cut into sections (1cm^2) under 10mM Hepes buffer (pH 7.0). The leaf sections (30 per treatment) were incubated in 10mM Hepes buffer (pH 7.0) alone or buffer containing 50 mM fructose, 50mM glucose, 50mM sucrose, 20mM H_2O_2, or 1mM methyl viologen. Leaf sections were incubated for 16 h in the dark in the above solutions, after which time the leaf sections were harvested for real time (qPCR) analysis.

Real time (qPCR) analysis

Purified total RNA was first treated with DNAse I (amplification grade; Invitrogen). The first strand cDNA was then prepared using SuperScript II (Invitrogen). Quantitative PCR was performed using this template. Sequences for ubiquitin, thioredoxin, and cyclophilin were selected as endogenous controls based on their equal expression values on microarray analysis. Primers were designed to amplify 50-53bp amplicons present in the probesets on the microarray chips. Quantitative PCR was performed at least in triplicate using the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, UK) and SYBR green as intercalating dye (Sigma, UK) with the following cycling profile: 1 x 50°C (2 min), 1 x 95°C (10 min), 40 x

[95°C (15 s), 60°C (1 min)]. Relative quantitative analysis of transcripts was performed using the Applied Biosystems Detection Software (SDS) v1.2.1. Relative expression values were confirmed in each case by at least two endogenous controls.

Isolation of full-length serine-type protease inhibitors

Total RNA was extracted from leaf three pooled samples from six maize seedlings at the five-leaf stage using the TriPure total RNA isolation kit with contaminant genomic DNA digested by RNase-free DNase. Total RNA (2 μg per sample) that had been subjected to reverse transcription using superscript III™ Reverse Transcriptase (RT) (Invitrogen, USA) was used as a template in the PCR reaction. Full-length cDNA clones for the *Zea mays* serine-type endopeptidase inhibitor (accession numbers EF406275) and Bowman-Birk-type serine protease inhibitor (accession number EF406276) were obtained by performing 5' and 3' rapid amplification of cDNA ends (RACE) using the GeneRacer™ kit (Invitrogen, USA) together with gene-specific primers. Gene-specific primers (forward 5'-tactcagctcaaggttgaaggcatgg–3' and reverse 5'-cgaatcacgcacactttggttcagag-3') were used for isolation of a full-length serine-type endopeptidase inhibitor and primers (forward 5'-cctcagctgatactcgtcggcact-3' and reverse 5'-gaacgtcgtcacagcggtaggtga-3') were used for isolation of a full-length Bowman-Birk-type serine proteinase inhibitor. The 5' RACE, 5' nested, 3' RACE and 3' nested primers were provided with the GeneRacer™ kit (Invitrogen, USA) that were used together with the gene specific primers. All amplified PCR products were T/A cloned into PCR4-TOPO (which was also provided with GeneRacer Kit) and sequenced in both direction using M13 forward and reverse primers. Inserts were sequenced using the BigDye® Terminator Cycle Sequencing FS Ready Reaction Kit, v 3.1 on ABI PRISM® 3100 automatic

DNA-Sequencer (Applied Biosystems, USA). The BLASTn and BLASTp programs (Altschul *et al*. 1990) were used for gene sequence homology search.

Analysis of putative serpin and BBI sequence isolated from maize

The cDNA sequences of EF406275 (putative serpin) and EF406276 (putative BBI) were analysed using online tools: BLASTx at GenBank (Altschul et al. 1990), WU-blastn V2.0 (http://blast.wustl.edu/; Gish & States 1993) on the EMBL database, ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), ProtParam (Gasteiger et al. 2005), and TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al. 2000).

Phylogenetic analysis of putative serpin and BBI sequences

After identification of their coding sequences, protein homologs of the putative serpin and putative BBI genes were identified by translated homology search (BLASTx) at GenBank. Translated gene sequences were compared to protein homologs by phylogenetic comparison, the alignments being determined by ClustalW (as implemented in the VectorNTI package), and the best unrooted tree was generated with PAUP4* in default parameter configuration and the resultant trees were displayed using the PhyloDraw package. Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number EF406275 and EF406276

Proteomic analysis

Leaf extracts prepared from leaf 3 and leaf 12 of eight week-old plants were prepared for 2D electrophoresis according to instructions in the handbook “2-D

Electrophoresis: Principles and Methods" (GE Healthcare). Three technical replicates were prepared from each extraction. Proteins were precipitated after grinding leaf material in liquid nitrogen. Ground leaf material (200-250mg) was incubated over-night at –20°C in precipitation buffer (1ml) containing TCA (10%, w/v) and β-mercaptoethanol (0.07% v/v) in acetone (100%, v/v). Precipitated protein was pelleted by centrifuging for 25 min at 4°C at 20 000xg and washed 6 times with ice cold washing buffer containing acetone (90%, v/v) and β-mercaptoethanol (0.07% v/v) in Milli-Q water. Proteins were solubilised in sample buffer (1ml) containing urea (8M), CHAPS (2%, w/v), DTT (60 mM), and IPG buffer pH 4-7 (0.5%, v/v) (GE Healthcare), by sonication in an ultrasonic water bath for 1 h, with vortexing at 15 min intervals. Samples were then incubated in a heating block for 1.5 h at 30ºC with vortexing at 15 min intervals before over-night incubation at room temperature for optimal protein solubilisation. Cell debris was removed by centrifugation for 25 min at 20,000xg. Solubilised proteins were quantified using the Bradford assay (Bradford 1976) and ovalbumin (Sigma) as standard (Ramagli 1999). 450µg of the protein extracts were diluted in 250µl rehydration buffer (7M urea, 2M thiourea, 0.75% (w/v) CHAPS, 0.75% (v/v) Triton X 100, 100mM DTT, a trace of bromophenol blue, 2% (v/v) IPG-buffer pH 4-7; GE Healthcare). Samples were then loaded onto 13cm immobiline DryStrip gels pH 4-7 (GE Healthcare) and run on an Ettan IPGphor II (GE Healthcare; rehydration, 20 h at 20ºC; 500 V for 1 h; 500 to 1000 V in 1 h; 1000 to 8,000 V in 2:30 h; and hold at 8,000 V for 55 minutes). IPG strips were then equilibrated first for 20 minutes in 50mM Tris-HCl (pH 8.8), 6M urea, 30% (w/v) glycerol, 2.3% (w/v) SDS, 1% (w/v) DTT, followed by 20 min in the same solution with DTT replaced with 4% (w/v) iodoacetamide. The IPG strips were then applied to 12.5 % resolving gels and were run in an SE 600 Ruby™, gel unit (GE Healthcare) at

25 mA gel^{-1} for 5 h at 10ºC. The running buffer was 25mM Trisbase, 192mM glycine, 0.1% SDS. Finally the gels were stained in a 0.1% solution of Phastgel-blue R-350 (GE Healthcare) in 40% methanol and 7% acetic acid overnight and de-stained in 40% methanol, 7% acetic acid, at room temperature on an orbital shaker. The stained gels were scanned on a calibrated Ettan Gel Imager (GE Healthcare) before being subjected to image analysis.

Image analysis

Progenesis SameSpots software (Nonlinear Dynamics) was used for gel analysis. The spots were matched between all the gels by using the SameSpots approach. Background subtraction and volume normalization were carried out. Artefacts and non-protein spots were removed by editing the template image and were expanded to all gels using the SameSpots option. The gels were also subjected to detailed Progenesis statistical analysis (t-test). Protein spots of interest were excised using Harris spot cutters (Sigma) before being subjected to in-gel digestion with trypsin and NANO-LC MS/MS analysis.

In-gel trypsin digest

Excised spots were washed with 25% (v/v) methanol and 7% (v/v) acetic acid for 12h at room temperature, and de-stained with 50mM NH_4HCO_3 in 50% (v/v) methanol for 1 h at 40°C. The gel pieces were then incubated in 10mM DTT, 100mM NH_4HCO_3 for 1 h at 60°C followed by 40mM iodoacetamide, 100mM NH_4HCO_3 for 30 minutes at room temperature. The gel pieces were minced, dried, rehydrated in 100mM NH_4HCO_3 containing 1pmol of trypsin (Promega) and incubated at 37°C overnight. The digested peptides were extracted from the gel slices with 0.1% TFA in 50% (v/v)

acetonitrile/water three times. The peptide solution was dried, re-suspended in 30µl of 0.1% TFA/5% acetonitrile/water.

LC-MS/MS

LC-MS/MS analysis was performed at NEPAF, Cels Ltd Newcastle. Using an Ultimate 3000 nano-HPLC system (Dionex LC Packings, Camberley, UK), 10% of the digest was injected at 20 µl /min onto a 300 µm x 50 mm PepMap trap column (Dionex). After washing with 60 µl of 0.1% formic acid at 20 µl /min, peptides were eluted through a 150 mm x 75 µm PepMap column (Dionex) at 200 nl/min. MS spectra were acquired on an HCT Proteome Discovery system (Bruker Daltonics, Bremen, Germany). A survey scan was conducted in standard enhanced mode (8100 Da/s) from m/z 400 to m/z 1600. The three most intense ions (excluding singly charged ions, absolute threshmature5000 counts) were fragmented and MS/MS scans were acquired in ultrascan mode (26000 Da/s) from 50 to 2100 Da. Parent ions were dynamically excluded for 90 s after two MS/MS acquisitions. Up to 300 000 ions were accumulated in the 3D trap with a maximum accumulation time of 120 s. Up to 750 spectra per LC/MS experiment were extracted from the data using Bruker DataAnalysis 3.4 software. MS/MS spectra were de-convoluted, converted to line spectra and exported as mgf files for homology searches. The translated *Oryza sativa* genome (as on 1 September 2008), the translated *Arabidopsis thaliana* genome (as on 1 September 2008) and a collection of frequently occurring contaminating proteins (common Repository of Adventitious Proteins, cRAP) and their reverse counterparts were searched using X!Tandem allowing for single amino acid substitutions and the gpm web interface at www.thegpm.org (Craig and Beavis, 2004). Search parameters

and results were submitted to the gpm database and can be accessed through the hyperlinked Identifier codes in the supplementary results table.

Statistical analysis

The statistical analysis of the micro-array data is described above. The gas exchange data was analyzed by ANOVA. Data for all other physiological parameters was analyzed by the Student's t-test or LSD test.

Accession numbers

Putative serine proteinase inhibitor sequence was submitted to GenBank (http://www.ncbi.nlm.nih.org) with accession number EF406275. Putative Bowman-Birk inhibitor sequence was submitted to GenBanks with accession number EF406276. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-1222.

Results

High CO_2 effects on whole plant morphology and photosynthesis

Maize plants were grown for up to eight weeks (Figure 1A) in either ambient CO_2 conditions (350+/- 20 μL L^{-1} CO_2) or with CO_2 enrichment (700+/- 20 μL L^{-1} CO_2). The plants grown with CO_2 enrichment exhibited a similar overall morphology to those grown under ambient CO_2 conditions (Figure 1A). In an earlier study we reported that the maize plants grown with CO_2 enrichment were taller than those grown in air although they had the same number of leaves (Driscoll *et al*., 2006). However, the more systematic analysis reported here revealed that there were small variations in the specifications of the controlled environment growth chambers used

previously and that these had led to differences in overall plant height (Driscoll *et al*. 2006). These small but important variations were eliminated in the present study by randomisation of chamber usage. The data presented here shows that growth with CO_2 enrichment has no effect on the height of the maize plants.

There were no significant differences in the fresh weights (Figure 1B), the dry weights (Figure 1C), or tissue water contents (Figure 1D) of leaves at equivalent positions on the stem. The leaf fresh weight values were similar regardless of ontogeny, but the dry weight values were greatest in the young leaves and decreased with leaf position on the stem (1 to 12). The leaves in ranks 1-5 had the lowest dry weight values (Figure 1C). Similarly, the tissue water content was greatest in leaf ranks 1-5 and decreased gradually with the leaf position on the stem, the young leaves having the lowest tissue water contents (Figure 1D). Specific leaf weight and specific leaf area values were similar under both conditions in all except the youngest leaves (Figure 1E and 1F). Leaf total anthocyanin contents were highest in leaf ranks 1-4, but values were similar in plants grown in ambient conditions or with CO_2 enrichment (Figure 1G).

Transpiration rates were similar at leaf rank 12 under both CO_2 conditions but they were decreased in the rank 5 leaves at high CO_2 (Figure 2A). Stomatal conductance rates were higher at rank 12 than rank 5, the difference being most marked in plants grown at high CO_2 (Figure 2B). Stomatal conductance was significantly higher at both leaf ranks under ambient CO_2 conditions compared to high CO_2 (Figure 2B). In line with the lower stomatal conductance rates at high CO_2, leaf temperatures were higher (Table 1).

The leaves at rank 12 had significantly higher (about 30%) rates of photosynthesis than those at leaf rank 5 under both CO_2 conditions (Figure 2C). However, photosynthesis rates were high at both leaf ranks and thus leaves at both ranks are denoted as "source leaves". While photosynthesis rates appeared to be lower at high CO_2 as observed previously (Driscoll *et al*., 2006), the differences between the two CO_2 conditions were not significant (Figure 2C). Photosynthesis rates were not significantly changed by the growth CO_2 environment (Figure 2C). Water use efficiency values were higher in leaves at rank 12 than rank 5 under ambient CO_2 conditions (Figure 2D). Growth with CO_2 enrichment significantly enhanced the water use efficiencies at both leaf ranks (Figure 2D). There were no significant differences in total C or total N contents or C/N ratios in leaves grown under ambient or high CO_2 conditions (Table 1).

The maize leaf transcriptome

The transcript profiles of leaf ranks 12 and 3 from plants grown under ambient and high CO_2 conditions were analysed. In each case, three plants were used for each RNA extraction pool (in line with the sample source being designated as 2-3 plants per sample per chip in microarray submission: E-MEXP-1222). The transcriptome profiles were similar under ambient and high CO_2 conditions and there were no significantly changed transcripts. In contrast, there were significant differences in transcriptomes of leaf ranks 3 and 12. This analysis revealed in 734 transcripts under ambient CO_2 conditions (Web Table 1) and significant differences in 738 transcripts with CO_2 enrichment (Web Table 2). Of these transcripts that were specifically changed by leaf ontogeny irrespective of the CO_2 enrichment, 569 transcripts were the

same under both CO_2 treatments (Figure 3 A). Leaf rank-dependent changes were observed in transcripts encoding proteins involved in nitrogen metabolism such as nitrate reductase, nitrate reductase and glutamine synthetase, which were higher in leaf rank 12 than 3. While mRNAs encoding sucrose phosphate synthetase, phospho*enol* pyruvate carboxylase and β amylase (Web Table 3) were higher in leaf rank 12 than 3, transcripts encoding sucrose synthase and UDPglucose dehydrogenase were lower in leaf rank 3 than 12 (Web Table 3). Transcripts encoding metacaspases, metallopeptidases, serine proteases and cysteine proteases and cysteine protease inhibitors were also higher in leaf rank 12 than 3 (Web Table 3). We also identified a putative serine protease inhibitor and a putative BBI serine protease inhibitor that were altered in a leaf rank-specific manner (Web Table 3). The full-length gene sequences of these inhibitors were obtained by RACE and compared to those of known similar inhibitors in the databases (Figures 3B, C and 4). The putative serine protease inhibitor EF406275 (serpin) was identified through homology to an Arabidopsis serine-type endopeptidase inhibitor (NP_177351.1; bit score 86.3; E=6e-16). The BBI sequence has a high sequence similarity to a patented maize sequence, which is described as a maize proteinase inhibitor-like polynucleotide (AR494954; patent number US6720480-A/1, 13-APR-2004).

Although no significant effects of atmospheric growth CO_2 level were found in the transcriptome analysis, the effects of growth CO_2 on the abundance of certain transcripts were also examined using qPCR. For this analysis we compared the transcripts encoding the two serine protease inhibitors and four invertase and sucrose synthase sequences associated with carbohydrate metabolism (cell wall invertase, acid invertase, two sucrose synthase forms, and sucrose phosphate synthase) in leaves of

plants grown either under ambient CO_2 conditions or with CO_2 enrichment. Under ambient CO_2 conditions the abundance of the serpin transcripts was dependent on leaf rank, with the highest levels in leaf ranks 1-3 (Figure 5A). The response of the BBI transcripts to growth with CO_2 enrichment was also dependent on the position of the leaf on the stem (Figure 5A and 5B). However, while serpin transcripts were increased as a result of growth with CO_2 enrichment only in the youngest source leaves (Figure 5A), BBI transcripts showed a CO_2 –dependent change in abundance only in the oldest source leaves (Figure 5B). While growth with CO_2 enrichment had no effect on the abundance of cell wall invertase, sucrose synthase, and sucrose phosphate synthase transcripts (Figure 5C-G), the abundance of acid invertase was higher in leaves of plants grown at high CO_2 (Figure 5C). The abundance of SPS transcripts was dependent on leaf rank with the highest levels measured in the youngest source leaves (Figure 5G). Less marked effects of leaf rank were found for transcripts encoding cell wall invertase, acid invertase, and the two sucrose synthase forms.

CO_2 –dependent effects on the leaf proteome

Leaf proteins extracted from leaf ranks 3 and 12 of ambient and high CO_2–grown maize plants were separated using 2-D gel electrophoresis (Web Table 4). Progenesis SameSpot 2D gel analysis software revealed the presence of about 900 spots in the leaf extracts. Relatively few spots were changed in abundance in rank 12 source leaves as a result of CO_2 enrichment. Growth with high CO_2 exerted the most pronounced effects on the proteome of rank 3 source leaves but the differences between differentially expressed proteins were generally less than 2-fold (Web Table 4). Sixty differentially-expressed spots were excised from the gels containing

proteins of leaf rank 3 leaves and subjected to LCMS/MS analysis as outlined in the materials and methods section. In total, 69 proteins were identified from 35 protein spots (Web table 4). Of these, 10 were increased in intensity and 25 spots were decreased in intensity in response to high CO_2 (Table 2). It is important to note that only 14 spot analyses resulted in the identification of a single protein, whereas the remaining 21 spots yielded between 2 and 7 different protein identifications per spot, making it impossible to decide which protein would have been responsible for the increase or decrease in intensity of the respective spot. However, the FtSH protease was identified in three different spots (23, 24 and 34), all of which showed an approximately 2-fold decrease in response to high CO_2. Many of the other identified proteins are involved in primary metabolism, particularly photosynthesis and respiration. The third largest functional group amongst the proteins altered in response to high CO_2 were enzymes involved in protein metabolism (Table 2).

Effects of sugars and cellular redox modulators on the abundance of maize leaf transcripts

The data shown in Figure 5 A, B, indicated that the abundance of transcripts encoding the serpin and BBI inhibitors were changed by the growth CO_2 level in a leaf rank specific manner. To determine whether the expression of serpin and BBI was influenced by sugars or subject to redox regulation, maize leaf pieces were incubated in solutions containing either sugars (sucrose, glucose or fructose) or pro-oxidants (hydrogen peroxide or methyl viologen; MV). While the sugars had very little effect on the abundance of the transcripts associated with sugar metabolism, incubation with sucrose, fructose or glucose increased the abundance of serpin transcripts and

decreased the levels of BBI transcripts (Table 3). Treatment with H_2O_2 and methyl viologen increased the levels of both serpin and BBI transcripts but caused a small decrease the abundance of transcripts associated with sugar metabolism (Table 3).

The effect of growth CO_2 levels on the leaf metabolome.

Growth with high CO_2 had very little effect on maize leaf metabolite profiles (Web Table 5). Significant differences were found in only 13 metabolites in leaf rank 5 of 9 week-old plants. No significant changes in photorespiratory metabolites such as glycolate, glycerate, glycine or serine were found. The levels of leaf glucose, mannose and galactose were enhanced as a result of growth with CO_2 enrichment as were the levels of linoleic and linolenic acids (Figure 6). Significant decreases were observed only in glutarate, 2-hydroxyglutarate, 2-oxoglutarate, alanine, proline, myo-inositol and two isomers of hydroxybenzoate (Figure 6).

The effect of growth CO_2 levels on the leaf-rank-specific accumulation of carbohydrate and on associated enzymes

High CO_2 had a marked effect on the leaf rank-specific profile of carbohydrates but there was little effect on the activities of key enzymes involved in carbohydrate metabolism (Figure 7). Glucose and fructose were much higher in the leaf ranks 1-5 compared to all other leaves on the stem, which had very low hexose contents when plants were grown under ambient CO_2 conditions (Figure 7A and 7B). Growth with CO_2 enrichment completely suppressed this leaf rank-dependent change in leaf hexoses such that all the leaves had similar low amounts of glucose and fructose in this condition (Figure 7A). Leaf sucrose (Figure 7C) and starch (Figure 7D) were not greatly changed as a result of leaf rank in plants grown under ambient CO_2 conditions.

However, there was a sharp increase in both of these carbohydrates in the leaf ranks 7-13 of plants grown with CO_2 enrichment.

Soluble acid invertase activities were much higher than those of neutral invertase in all but leaf ranks 10-13 of plants on the stem (Figure 7 E). While neutral and acid invertase activities were unaffected by growth CO_2 level (Figure 7 E), SPS activities were markedly increased as a result of growth with CO_2 enrichment, in all but leaf ranks 1-3 and 10-13 (Figure 7 F).

The effect of growth CO_2 level on the leaf-rank-specific accumulation of protein carbonyl groups

The extent of protein carbonyl group formation is controlled by leaf development in Arabidopsis (Johansson, Olsson & Nystrom 2004) but it is often used as a measure of cellular oxidation, as it increases in response to stress (Kingston-Smith & Foyer 2000). Leaf rank specific changes in the content of protein carbonyl group formation were apparent in maize (Figure 8) like Arabidopsis leaves (Johansson *et al*. 2004). However, in the case of maize, protein carbonyls were most abundant in leaves 9-12 and they decreased progressively to leaf rank 1, which showed only one detectable band of carbonyl groups in plants grown under ambient CO_2 conditions (Figure 8A). The intensity of this single carbonyl-stained protein in leaf ranks 1 and 2 was increased in plants grown at high CO_2 (Figure 8B). While growth with CO_2 enrichment had a dramatic effect on the profile of leaf carbonyl abundance and composition, the effect varied with leaf rank. The number of protein bands showing staining for protein carbonyl group was decreased in leaves 9-12 (Figure 8B). In particular, there was a marked decrease in the number of high molecular weight

proteins showing carbonyl group formation in leaves 9-12 at high CO_2 (Figure 8). With exception of leaf ranks 1-4, the level of protein carbonyl formation was also modified in other leaf ranks but this effect was highly dependent on the molecular weight band analyzed (Figure 8).

Discussion

While future increases in atmospheric CO_2 availability will benefit plants with the C_3 photosynthetic pathway, the nature of the responses of C_4 plants to elevated CO_2 remains controversial (Leakey, 2009). Evidence from the Free-Air Concentration Enrichment Experiments (FACE) has consistently shown that growth with elevated CO_2 does not enhance maize leaf photosynthesis or plant growth (Leakey *et al*. 2006; 2009 a,b). The results presented here confirm the observations from the FACE experiments and also data from other similar studies (Kim *et al*. 2006). Similarly, the leaf transcriptome analysis failed to show any significant differences between the air and high CO_2 samples in line with earlier observations (Kim *et al*. 2006; Leakey *et al*. 2009 a, b). However, further analysis using qPCR revealed that transcripts encoding a BBI and a serpin were differentially expressed in relation to the growth CO_2 level in a leaf rank-specific manner. Similarly, the leaf proteome analysis revealed that several chloroplast proteins including components of the PSII oxygen evolving complex and the ATP synthase were decreased at high CO_2 but only in the older source leaves. Of the proteins that were altered in abundance at high CO_2, there was a marked effect on FtsH proteases, which are important in chloroplast biogenesis and thylakoid maintenance. The high CO_2–dependent decreases in FtsH proteases may reflect the diminished requirement for photosystem repair in the older source leaves at high

CO_2compared to ambient CO_2. These modest adjustments in chloroplast components were not however sufficient to cause a significant decrease in the overall rate of leaf photosynthesis. Analysis of the leaf metabolome showed that while metabolites associated with photorespiration were similar in leaves under ambient and high CO_2 conditions, the levels of some carbohydrates such as glucose, mannose and galactose were enhanced in the older source leaves as a result of growth with CO_2 enrichment. Similarly, spectrophotometric measurements of leaf starch, sucrose, glucose and fructose showed that these metabolites were modified as a result of growth with CO_2 enrichment but that changes in abundance were dependent on the leaf position on the stem. Taken together, the data suggest that maize leaves show acclimation to CO_2 enrichment but that this occurs in a strict leaf-specific manner. Leaf ontogeny has a marked influence on leaf responses to atmospheric CO_2 availability. Maize leaves undergo a shift in photosynthesis from C_3 to C_4 metabolism during development (Crespo *et al*. 1979) and large number of transcripts were significantly changed in abundance between leaf ranks 3 and 12.

A key question that remains to be addressed concerns whether leaves are able to sense and signal changes in atmospheric CO_2 by pathways that are independent of photosynthesis. One mechanism that has been proposed implicates carbonic anhydrases in plant CO_2-signalling pathways (Hu *et al*. 2010). While the data presented here do not provide any new insights into the roles of carbonic anhydrases in the CO_2-signalling pathways in maize, the data clearly demonstrate that the acclimation responses to high CO_2 observed in maize are dependent on leaf rank. Thus, we explored how different parameters associated with leaf physiology were affected by growth at ambient and high CO_2. The most pronounced effects were

observed in the leaf transpiration rates, which were decreased in leaves grown at high CO_2 (Figure 2). As a consequence of decreased transpiration rates, the leaf temperature were slightly increased in plants grown at high CO_2 (Table 1). However, the overall changes in maize leaf temperatures are very small, as observed previously (Kim *et al*. 2006). Thus, it is unlikely that CO_2-dependent changes in leaf temperature are responsible for the observed changes in the leaf proteins, transcripts and metabolites. It has previously been suggested that high CO_2–dependent changes in leaf metabolism are related to altered leaf N status (Kim *et al*. (2006). However, in the present experiments neither maize leaf N status nor leaf C/N ratios were significantly changed as a result of growth at high CO_2.

Drought stress can be ameliorated at elevated CO_2 even in C_4 plants as a result of lower stomatal conductance and thus it is possible that maize leaves may be much less susceptible to water deficits when grown with CO_2 enrichment. Thus, the observed differences in transcripts, proteins and metabolites between the somewhat less photosynthetically-active leaves (ranks 3-5) and the younger (rank 12) source leaves may be related to slight changes in leaf water status. The stomatal conductance rates were significantly higher in leaves under ambient CO_2 conditions (Figure 2B). While transpiration rates (Figure 2A) and water use efficiency values (Figure 2D) were similar in leaf rank 12 under both CO_2 conditions, the differences were significant in the older less photosynthetically active leaves (rank 5). The small CO_2–dependent differences in the water status of the rank 12 leaves could therefore be responsible for the observed variations in transcripts, proteins and metabolites. The decreased abundance of myo-inositol and two hydroxybenzoic acids, which are related to SA metabolism, were repressed at high CO_2 and there was a decreased abundance of

protein carbonyl groups. These findings are consistent with a lower oxidative load in leaves grown under high CO_2 conditions. This may be particularly important for bundle sheath proteins, which are more sensitive to carbonylation in response to cellular oxidation than those of the mesophyll (Kingston-Smith & Foyer, 2000). The lower levels of protein oxidation under high CO_2 conditions are indicative of an altered cellular redox status, particularly in the younger leaf ranks. Alterations in cellular redox status may have important implications for enzymes and metabolic processes such as starch metabolism (Hendriks *et al*. 2003; Kolbe *et al*. 2005; Sparla *et al*. 2006) that are subject to redox regulation (Buchanan & Balmer 2005; Schürmann & Buchanan 2008).

Growth with high CO_2 exerted a marked influence on leaf carbohydrate status in a strict leaf-rank specific manner and this also might be related to changes in whole plant water status. A CO_2–dependent accumulation of sucrose was observed only in the youngest leaves (ranks 9-12) but high CO_2 prevented the accumulation of hexoses in the older (ranks 1-3) leaves. These changes in the leaf carbohydrate profiles occurred in the absence of any detectable changes in the activities of key enzymes involved in carbohydrate metabolism but it might be related to decreased assimilate export from the younger leaves at high CO_2. Interestingly, sugars are involved systemic signalling pathways that convey information concerning CO_2 availability from leaf to leaf (Coupe *et al*. 2006; Miyazawa *et al*. 2006; Baena-González *et al*. 2007). The failure of the older leaves to accumulate hexoses when grown under high CO_2 might also be related to decreased sucrose export from the younger leaves, particularly if this was associated with futile cycling between sucrose and hexoses (Nguyen-Quoc & Foyer, 2001).

The functions of BBI and the serpin that were differentially expressed in relation to the growth CO_2 level in a leaf rank-specific manner are not known. The serpin (EF406275) is homologous to an Arabidopsis serine-type endopeptidase inhibitor (AT1G72060) and has signal peptide and pin2 domains. The BBI protein has a theoretical molecular weight of 10.4 kDa and pI of 6.01 with signal peptide and BB leg domains. The signal peptides suggest that both proteins are targeted to classical secretory pathways and therefore may be involved in autophagocytotic pathways of protein degradation associated with the vesicular transport system or with processes located in the extracellular/apoplastic space. The vesicular trafficking system has functions linked to plant stress responses (Leshem *et al*. 2006) and to the degradation of Rubisco and other chloroplast proteins (Chiba *et al*. 2003; Ishida *et al*. 2007; Prins *et al*. 2008).

While plant serpins have been intensively studied particularly in cereal seeds, relatively few protein targets have been identified to date and precise roles have yet to be assigned. One target has been shown to be the cysteine-dependent protease, metacaspase 9, which is strongly inhibited by Arabidopsis Serpin1. However, the *in vitro* inhibition of trypsin and metacaspase 4 imply that Serpin1 has a range of targets (Vercammen *et al*. 2006).

The serpin and BBI transcripts were most abundant in the older leaf ranks 1-3, which is consistent with roles in autophagocytotic pathways of protein degradation. Their expression patterns of these protease inhibitors were changed by growth at high CO_2 in the older source leaves and further examination revealed that the abundance of the

transcripts was modulated in response to sugars and redox effectors. The serpin transcripts were increased in the presence of sugars while the BBI transcripts were repressed under these conditions. The abundance of serpin transcripts was lower in the older source leaves grown at high CO_2, consistent with the high CO_2–dependent decease in leaf hexoses. These results may implicate sugar signalling in the regulation of serpin expression at high CO_2. However, CO_2–dependent alterations in sugar signalling do not appear to be involved in the regulation of the expression of the BBI inhibitor.

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Legends to Figures

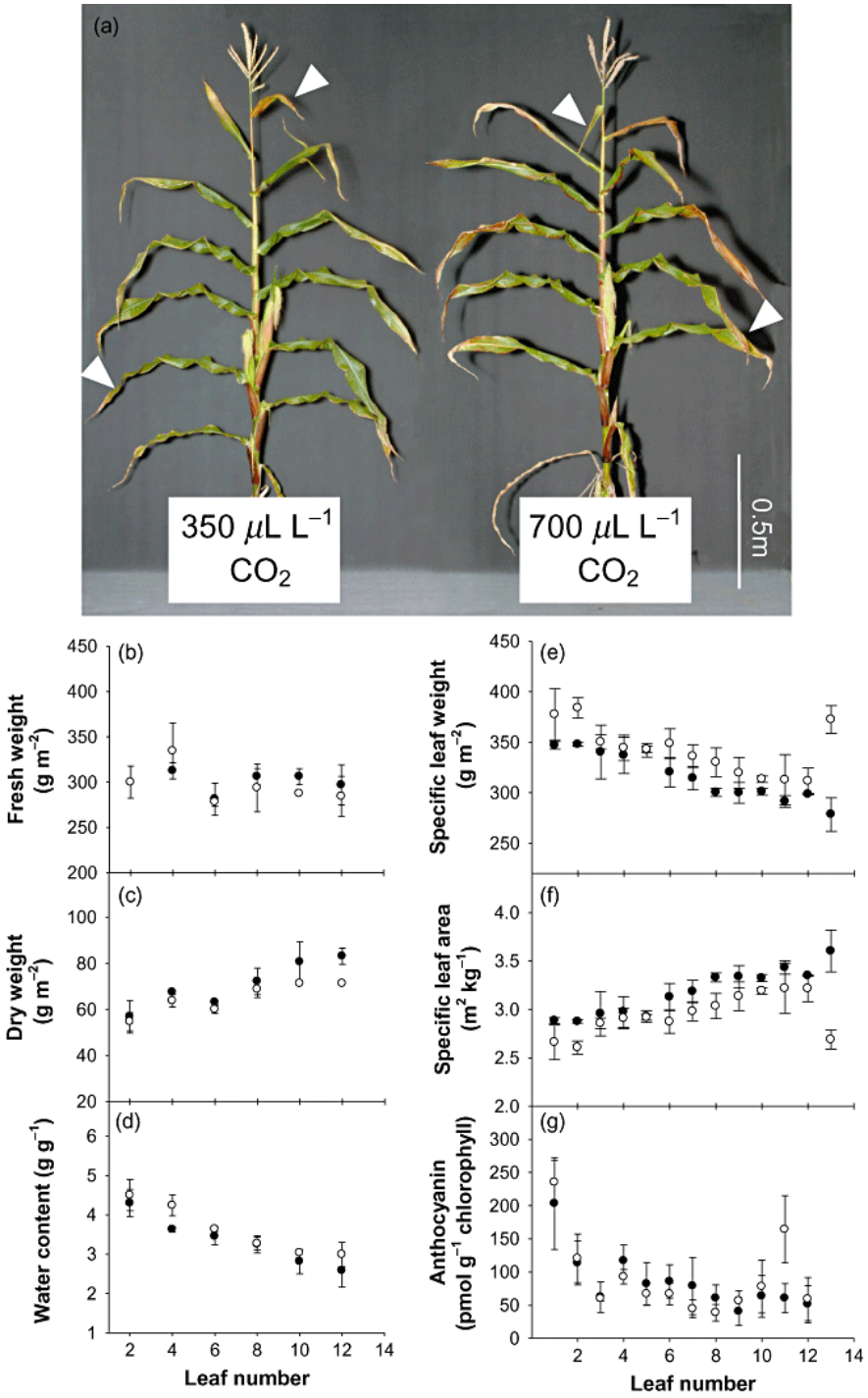

Figure 1. A comparison of the morphologies of eight week-old maize plants grown under either ambient CO_2 conditions (350 μL L^{-1}) or with CO_2 enrichment (700 μL L^{-1}). Whole plant phenotype (A) showing the ranking of all leaves on the stem. Samples were harvested from plants grown under either ambient CO_2 conditions (closed circles) or with CO_2 enrichment (open circles). Fresh weight (B) and dry weight (C) values were used to calculate tissue water content (D). Specific leaf weight (E). Specific leaf area (F). Leaf anthocyanin content (G). Significant differences at $P < 0.05$ indicated by the symbol (*).

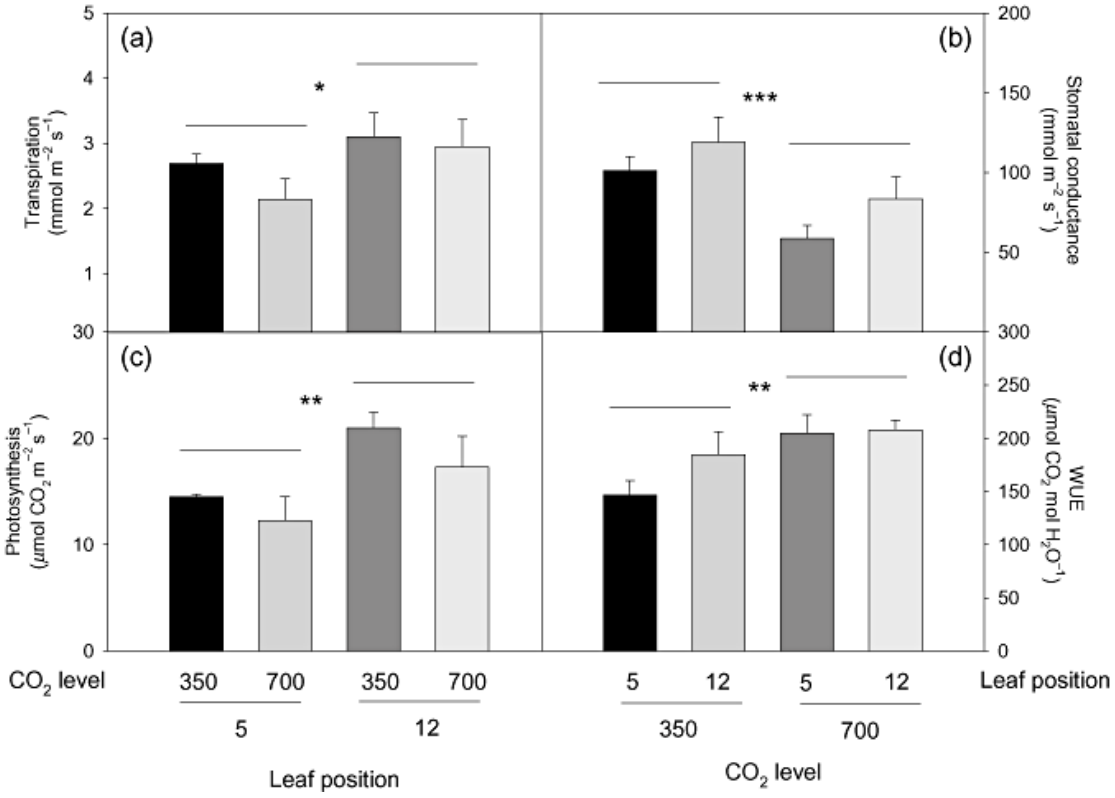

Figure 2. Comparison of transpiration rates (A), stomatal conductance (B) photosynthesis (C) and water use efficiencies (WUE, D) in source leaves at ranks 5 and 12 on plants that had been grown for eight weeks under either ambient CO_2 conditions (350+/- 20 µL L^{-1}) or with CO_2 enrichment (700+/- 20 µL L^{-1}). Values represent mean ± SE; n=4. Data were analysed by two-factorial ANOVA and significant differences between treatments are displayed (* at 10%, ** at 5%, *** at 1%)

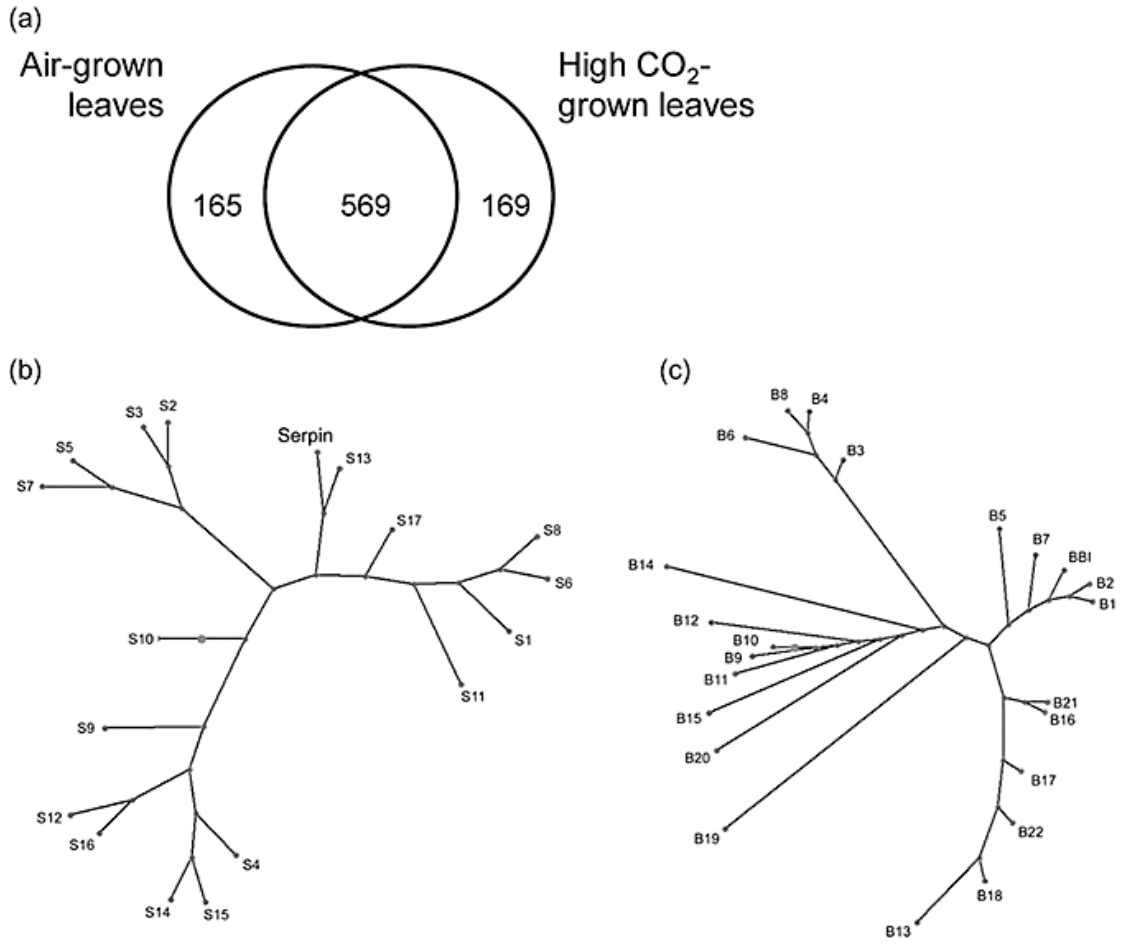

Figure 3. A comparison of transcripts responsive to leaf ontogeny under either ambient CO_2 conditions (Air-grown leaves) or with CO_2 enrichment (High CO_2-grown leaves; A). Phylogenetic trees showing the relationships between putative serpin and known serine proteinase inhibitor protein sequences (S1-S17; B) and putative BBI and known Bowman-Birk serine protease inhibitor protein sequences (B1-B22, B) listed in (C).

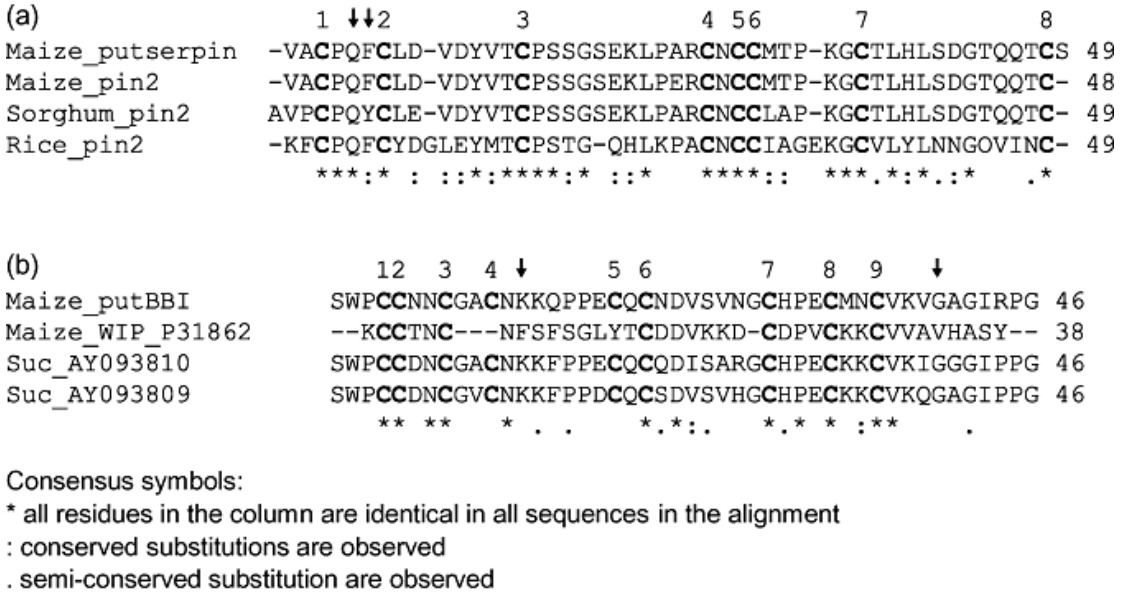

Figure 4. Multiple alignment of the identified serine proteinase inhibitor protein sequences with those of known serine proteinase inhibitors available in the database. Maize_putserpin – putative maize serpin (EF406275). Maize_pin2 (AI947362), Sorghum_pin2 (AI724716), Rice_pin2 (AU163886) – serine protease inhibitors identified in maize, sorghum, and rice respectively. Maize_putBBI - putative maize BBI (EF406276). Suc_AY093810 and Suc_AY093809 - two BBI sequences identified in *S. officinarum.* Maize_WIP_P31862 - a wound-induced protein from maize. Conserved cysteine residues that participate in disulphide bridges are numbered. Arrows indicate the putative protease-contact residues (Barta *et al*. 2002; Mello *et al*. 2003). Consensus symbols indicate: ***** - all residues in the column are identical in all sequences in the alignment; **:** - conserved substitutions are observed; **.** – semi-conserved substitutions are observed.

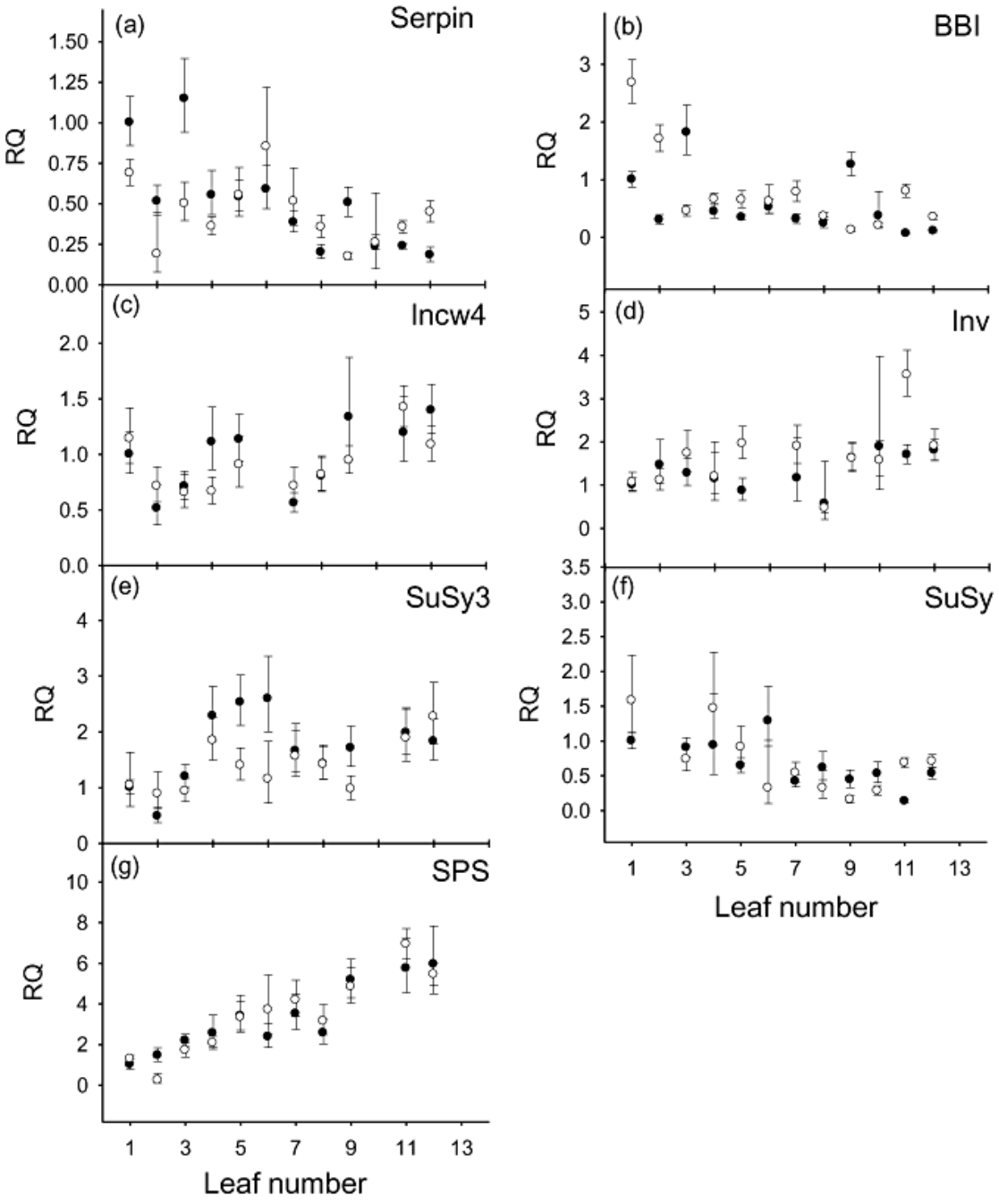

Figure 5. The effects of CO_2 enrichment on leaf rank-specific transcript levels measured by qPCR. Plants were grown for eight weeks under either ambient CO_2 conditions (closed circles) or with CO_2 enrichment (open circles). The abundance of transcripts encoding seprin (A) BBI (B) cell wall invertase (C), invertase (D), sucrose synthase 3 (E), sucrose synthase (F), and sucrose phosphate synthase (G) was determined in all the leaves. Values ± max/min are normalized to leaf 1 grown under ambient CO_2 conditions (RQ = 1) and expressed relative to endogenous controls.

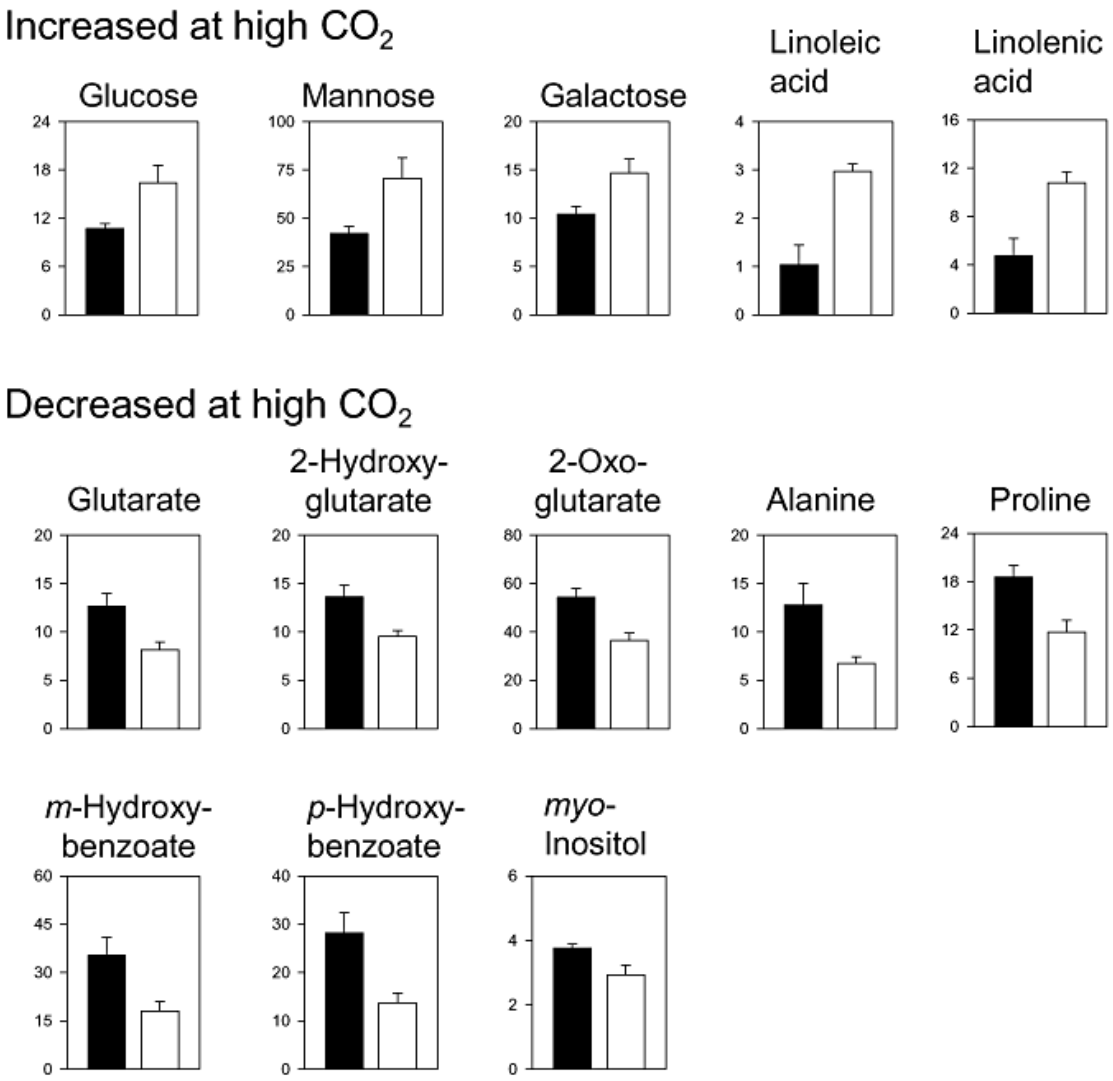

Figure 6. Effects of high CO_2 on maize leaf metabolite profiles. Plants were grown for six weeks under either ambient CO_2 conditions or with CO_2 enrichment. For a full data list, see Web Table 5. Note that peak intensities are in arbitrary units for each metabolite. Black bars, air. White bars, high CO_2. Data are means ± SE (n = 4).

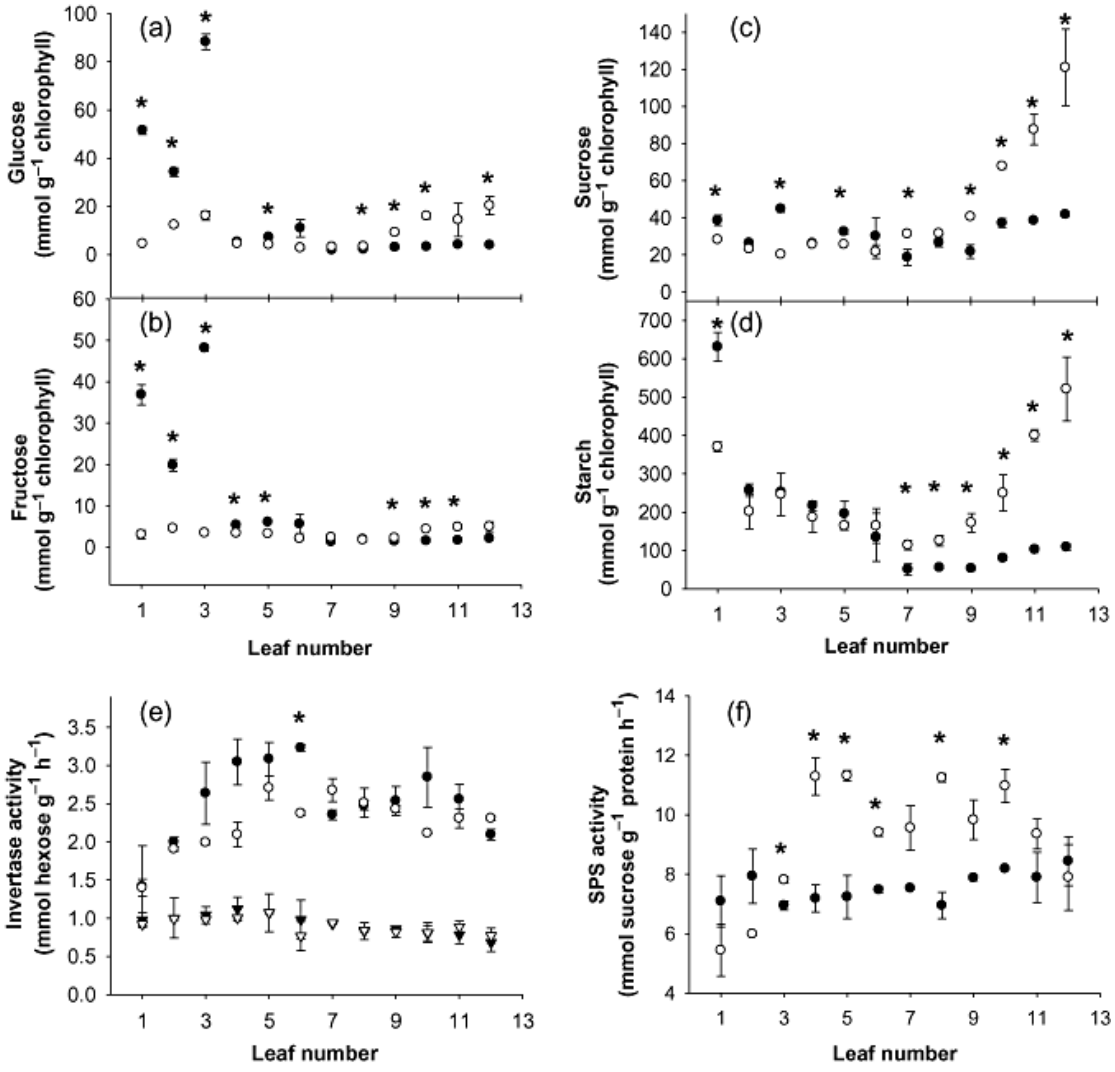

Figure 7. The effects of CO_2 enrichment on leaf rank-specific levels of hexoses, sucrose and starch and activities of sucrose phosphate synthase and invertase. Plants were grown for eight weeks under either ambient CO_2 conditions (closed circles) or with CO_2 enrichment (open circles). Glucose (A) fructose (B) sucrose (C) and starch (D) were determined in all the leaves as were the activities of sucrose phosphate synthase (D) and acid invertase (E). Activities of neutral invertase were also determined (E) in leaves under ambient CO_2 conditions (closed symbols) or with CO_2 enrichment (open symbols). Significant differences at $P < 0.05$ indicated by the symbol (*).

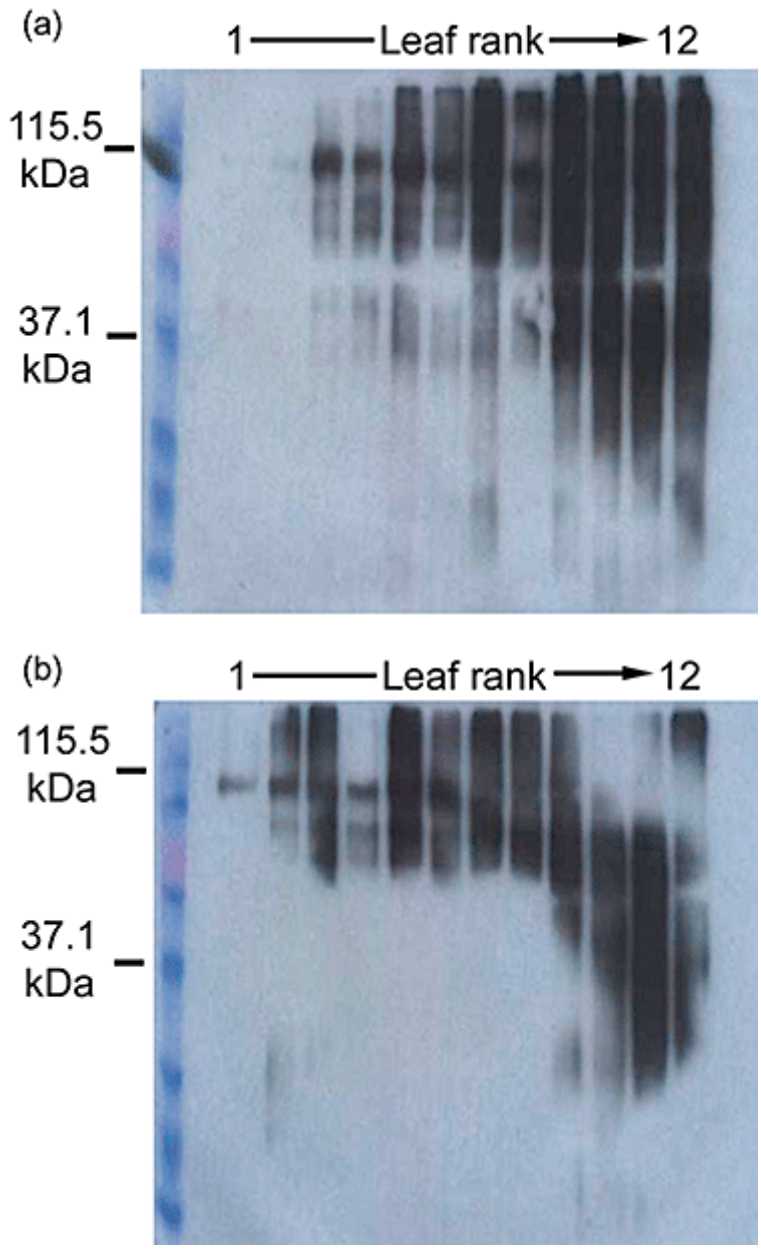

Figure 8. The effects of CO_2 enrichment on the leaf rank-specific abundance of protein carbonyl groups. Plants were grown for eight weeks under either ambient CO_2 conditions (A) or with CO_2 enrichment (B). Protein carbonyl groups are compared for all leaves on the stem from leaf rank 1 to leaf rank 12.

Table 1. A comparison of leaf C and N contents (% of dry weight), leaf C/N ratios and leaf temperatures (T_{leaf} in ^{o}C) in three week-old maize plants grown either under ambient CO_2 conditions or with CO_2 enrichment. Values represent mean ± SE; n=4. Superscript letters are significantly different ($P<0.05$).

	ambient CO_2	CO_2 enrichment
C (%)	41.72 ± 0.41^{a}	41.64 ± 0.05^{a}
N (%)	3.23 ± 0.09^{a}	3.06 ± 0.30^{a}
C/N	12.94 ± 0.23^{a}	13.72 ± 1.40^{a}
T_{leaf} at 700 µL L^{-1}	27.77 ± 0.41^{b}	28.80 ± 0.17^{a}
T_{leaf} at 380 µL L^{-1}	27.60 ± 0.36^{b}	28.63 ± 0.25^{a}

Table 2. Proteins involved in protein metabolism that occur in 2DE spots of which the intensity is modulated by CO_2 enrichment at leaf rank 3 of plants grown for eight weeks under either ambient CO_2 conditions (350+/- 20 $\mu L\ L^{-1}$) or with CO_2 enrichment (700+/- 20 $\mu L\ L^{-1}$) ..

Column titles are: ratio CO_2[High]/CO_2[Low]: -fold change of spot intensities as measured from 2D gel; Spot no: Spot number as numbered on 2D gel; migration 1st dim [cm]: migration of the protein in the isoelectric focussing dimension on the 2D gel, migration 2nd dim [cm]: migration of the protein in the SDS-PAGE dimension on the 2D gel; Identifier: Database acccession code of spot homologue; log(I): base 10 logarithm of the sum of the intensities of all the MS S spectra that contributed to the protein identification event; log(e): base 10 logarithm of the probability that a protein identification is a random event; pI: predicted isoelectric point of an identified protein; Mr: predicted molecular weight of an identified protein; Description: text based description of an identified protein obtained from the database search or a BLAST-based homology search

ratio CO_2[High] /CO_2[Low]	Spot No	migration 1st dim [cm]	migration 2nd dim [cm]	Identifier	single protein ID in spot	log(I)	rI	log(e)	pI	Mr [kDa]	Description
1.95	27	5.1	7.8	LOC_Os08g03640.1		6.57	4	-4.6	5.4	34.4	60S acidic ribosomal protein P0 {Oryza sativa (japonica cult
1.45	92	1.8	8.8	LOC_Os04g55650.1	√	5.69	1	-7.8	5.7	28.4	cysteine proteinase RD21a precursor, putative, expressed
-1.45	91	10.5	5.9	LOC_Os02g55420.1		5.51	1	-3.0	8.2	50.3	aspartate aminotransferase, chloroplast precursor, putative, expressed
-1.46	89	7.8	7.5	LOC_Os08g03640.1		5.42	1	-3.9	5.4	34.4	60S acidic ribosomal protein P0 {Oryza sativa (japonica cult
-1.46	87	9.6	4.9	LOC_Os01g36890.1		5.07	1	-3.0	5.5	48.6	spliceosome RNA helicase BAT1, putative, expressed
-1.61	49	14	6.2	LOC_Os02g55420.1		5.40	1	-7.2	8.2	50.3	aspartate aminotransferase,

											chloroplast precursor, putative, expressed
-1.67	48	7.3	5.4	LOC_Os02g05330.1		6.56	7	-55.7	5.4	47.1	eukaryotic initiation factor 4A, putative, expressed
-1.67	48	7.3	5.4	LOC_Os01g71270.1		5.54	1	-4.4	5.3	49.0	Eukaryotic peptide chain release factor subunit 1-2, putative, expressed
-1.74	42	9.6	3.2	LOC_Os06g36700.1		5.67	2	-10.8	5.7	59.1	T-complex protein 1 subunit epsilon, putative, expressed
-1.81	34	6.4	3.2	LOC_Os06g45820.1	√	6.79	14	-115.0	5.5	72.5	OsFtsH2 - Oryza sativa FtsH protease, homologue of AtFtsH2/8, expressed
-1.99	24	6.8	3.2	LOC_Os06g45820.1	√	7.03	9	-64.0	5.5	72.5	OsFtsH2 - Oryza sativa FtsH protease, homologue of AtFtsH2/8, expressed
-2	23	7.1	3.2	LOC_Os06g45820.1		6.01	6	-55.3	5.5	72.5	OsFtsH2 - Oryza sativa FtsH protease, homologue of AtFtsH2/8, expressed
-2.29	16	10.5	2.3	LOC_Os01g01830.1		5.53	1	-6.1	5.3	82.0	prolyl endopeptidase, putative, expressed

Table 3. Effects of sugars and pro-oxidants on the abundance of serpin and BBI transcripts and on selected transcripts encoding enzymes involved in sugar metabolism. Data represent relative minimum-maximum values calculated from at least 3 technical replicates normalised to values obtained from leaves incubated with buffer alone and relative to cyclophilin and ubiquitin as endogenous controls.

		Treatment				
		Sucrose	**Fructose**	**Glucose**	**H_2O_2**	**MV**
Transcript	**Serpin**	1.83	1.59	1.44	2.91	7.56
		(1.64-2.04)	(1.19-2.12)	(1.34-1.54)	(2.51-3.38)	(6.05-9.47)
	BBI	0.54	0.52	0.44	1.97	1.32
		(0.48-0.61)	(0.32-0.85)	(0.38-0.51)	(1.71-2.27)	(1.15-1.52)
	Incw4	0.96	0.95	0.72	0.78	0.91
		(0.86-1.07)	(0.79-1.13)	(0.67-0.78)	(0.66-0.92)	(0.76-1.10)
	Inv	0.82	1.31	1.04	0.32	0.66
		(0.54-1.24)	(0.96-1.80)	(0.89-1.21)	(0.24-0.42)	(0.56-0.78)
	SuSy3	1.12	1.29	1.17	0.45	1.70
		(0.94-1.33)	(1.07-1.55)	(1.03-1.34)	(0.32-0.64)	(1.52-1.89)
	SuSy	1.22	1.07	0.72	1.81	0.78
		(0.99-1.49)	(0.72-1.60)	(0.67-0.77)	(1.44-2.28)	(0.62-0.98)
	SPS	0.91	1.18	0.76	0.30	0.77
		(0.74-1.11)	(1.00-1.40)	(0.72-0.81)	(0.25-0.35)	(0.67-0.87)