Canopy photosynthesis, evapotranspiration, leaf nitrogen, and transcription profiles of maize in response to CO₂ enrichment

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Abstract

The effects of CO₂ enrichment on the growth and physiology of maize were investigated at the molecular, biochemical, leaf, and canopy levels. Maize plants were grown in sunlit soil–plant–atmosphere research (SPAR) chambers at ambient (370 \( \mu \text{mol mol}^{-1} \)) or elevated (750 \( \mu \text{mol mol}^{-1} \)) atmospheric carbon dioxide concentration \((C_a)\) under well-watered and fertilized conditions. Canopy gas exchange rates and leaf temperatures were monitored continuously during the growing season. CO₂ enrichment did not enhance the growth or canopy photosynthesis of maize plants. However, canopy evapotranspiration rates decreased by 22% and daytime leaf temperatures were increased about 1°C in response to CO₂ enrichment. Leaf carboxylation efficiency and leaf nitrogen concentration also decreased at elevated \( C_a \). Transcription profiling using maize cDNA microarrays revealed that approximately 5% of tested genes responded to CO₂ enrichment. Of the altered transcripts, several were known to encode proteins involved in stomatal development or photosynthesis. For the majority of the altered transcripts, however, it was difficult to link their functions with specific physiological factors partly because many of these genes encoded unknown proteins. We conclude that maize did not exhibit enhanced growth or photosynthesis in response to CO₂ enrichment but a number of molecular and physiological processes including those involved in stomatal relations were affected by growth in elevated \( C_a \).

Keywords: C₄ photosynthesis, canopy gas exchange, cDNA microarrays, CO₂ enrichment, leaf temperature, stomata, sunlit growth chambers, transpiration, Zea mays L.

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Introduction

Global atmospheric CO₂ concentrations \((C_a)\) are rising (367 \( \mu \text{mol mol}^{-1} \) in 1999) and are projected to reach 540–970 \( \mu \text{mol mol}^{-1} \) by the end of the 21st century (Prentice et al., 2001). While a number of studies dealt with the effects of elevated \( C_a \) on the physiological and biochemical processes in plants, the majority of prior studies focused on \( C_3 \) species that are known to be most sensitive to changes in \( C_a \) (see reviews by Kimball et al., 2002; Long et al., 2004; Nowak et al., 2004). About 18% of global primary productivity is contributed by \( C_4 \) plants (Ehleringer et al., 1997). Hence, any significant changes in \( C_4 \) productivity in response to rising \( C_a \) and other climatic variables are likely to have a substantial impact on global primary productivity (Wand et al., 1999). Although the photosynthetic and growth responses to elevated \( C_a \) in \( C_4 \) plants were expected to be minimal owing to the biochemical and anatomical specialization associated with the CO₂ concentrating mechanism based on \( C_4 \) acid metabolism, enhanced photosynthesis and growth have been observed in response to elevated \( C_a \) in \( C_4 \) species (Wand et al., 1999; Ghanoum et al., 2000). Maize, a NADP-ME-type \( C_4 \) species, is the most widely cultivated \( C_4 \) crop ranking as the third most produced food crop in the world (Young & Long, 2000). Any effects of elevated \( C_a \) on
maize are likely to have significant consequences in terms of global food production (Leakey et al., 2004). Thus, it is critical to accurately assess how elevated \( C_\text{a} \) will affect growth and physiology of maize.

Enhanced plant growth at high \( C_\text{a} \) in \( C_4 \) species could be partly attributable to improved water use efficiency (WUE) under water-limited conditions (Ghannoum et al., 2000). Studies of \( C_4 \) plants (e.g. Wall et al., 2001; Leakey et al., 2004) showed that photosynthesis and/or growth were improved under elevated \( C_\text{a} \) when plants were subjected to transient or moderate drought stress. Whether CO2 enrichment enhances growth and photosynthesis of \( C_4 \) plants under well watered and fertilized conditions remains controversial (Wong, 1979; Hocking & Meyer, 1991; Samarakoon & Gifford, 1996; Maroco et al., 1999). A key limitation in most prior studies of \( C_4 \) plant responses to elevated \( C_\text{a} \) was that photosynthetic rates have been measured at the single leaf level over the short term. Short-term analyses of steady-state leaf photosynthesis (\( A_{\text{leaf}} \)) of mature leaves do not fully account for season long whole plant carbon dynamics, which is further complicated by such factors as respiration, leaf expansion, and root-shoot partitioning (Le-Cain & Morgan, 1998; Ghannoum et al., 2000). Leaf photosynthesis measurements are often poorly correlated with either biomass accumulation or crop yield (Nelson, 1988), whereas there is generally good correlation between season long canopy photosynthesis measurements and yield (Zelitch, 1982). It is also possible that enhanced rates of \( A_{\text{leaf}} \) under elevated \( C_\text{a} \) may be apparent only during early developmental stages in \( C_4 \) plants (Cousins et al., 2001). Enhanced growth at high \( C_\text{a} \) in \( C_4 \) plants might result from changes in diurnal CO2 assimilation patterns (Ghannoum et al., 2000). Therefore, the most accurate method to relate photosynthesis and growth responses to elevated \( C_\text{a} \) is to quantify diurnal variations in canopy CO2 assimilation rates from early development to a fully closed canopy.

Elevated \( C_\text{a} \) has been shown to reduce leaf nitrogen in \( C_3 \) plants (Conroy & Hocking, 1993; Stitt & Krapp, 1999; Gifford et al., 2000) and the interaction between elevated \( C_\text{a} \) and nitrogen was seen to be closely related to acclimation of photosynthesis (Stitt & Krapp, 1999). Leaf nitrogen also decreased by 6% under elevated \( C_\text{a} \) in several \( C_4 \) grass species (Wand et al., 1999). Reduced nitrogen uptake because of lowered transpiration rates at high \( C_\text{a} \) may reduce leaf nitrogen level in response to CO2 enrichment (Conroy & Hocking, 1993; McDonald et al., 2002). Growth in elevated \( C_\text{a} \) may result in morphological changes such as lower stomatal density (Maroco et al., 1999; Lake et al., 2001), increased tillering (Hocking & Meyer, 1991), or enhanced leaf area (Wand et al., 1999) without concommitant changes in leaf photosynthetic rates. For example, stomatal development of new leaves was down-regulated by CO2 sensing of mature leaves exposed to high \( C_\text{a} \) in Arabidopsis (Lake et al., 2001). Specific genes may regulate phenotypic responses of plants to elevated \( C_\text{a} \) with or without the direct involvement of photosynthesis. The cDNA microarray technology is a useful tool for studying plant responses to such environmental treatment as high \( C_\text{a} \) or drought at the gene level (e.g. Yu & Setter, 2003; Miyazaki et al., 2004).

In the present study, we tested if CO2 enrichment altered growth and physiology of \( C_4 \) maize at the molecular, biochemical, leaf, and canopy scales. We investigated transcriptome profiles of maize leaf tissue to identify genes that are responsive to CO2 enrichment. We monitored leaf temperature and gas exchange of maize plants at the canopy level throughout the experiment. We also measured leaf gas exchange rates, leaf carbon and nitrogen content, and \( C_4 \) enzyme activities to examine the physiological and biochemical mechanisms underlying the canopy responses to elevated \( C_\text{a} \).

Materials and methods

**Plant culture**

Maize plants (Zea mays L. cv. Pioneer 3733) were grown from individual seeds in sunlit soil–plant–atmosphere research (SPAR) chambers located at the Beltsville Agricultural Research Center (Beltsville, MD, USA). A physical description of these SPAR chambers and methods of operation and monitoring have been described previously (Baker et al., 2004). Six SPAR chambers were randomly assigned to ambient (370 \( \mu \text{mol mol}^{-1} \)) or elevated \( C_\text{a} \) (750 \( \mu \text{mol mol}^{-1} \)) treatment. Plants were grown on a mix of sterilized sand and vermiculite (1:1 by volume) in a soilbin with a volume of 1 m³ (2 m × 0.5 m × 1 m). Thirty-two seeds per chamber were sown on 20 August 2002. All chambers were controlled at 31/25 °C temperatures with 16 h of 31°C from 05:00 to 21:00 hours eastern standard time. The seedlings were thinned to 16 plants per chamber with a 25 cm × 25 cm spacing 8 days after planting (DAP). Plants were fertigated four times daily for 5–15 min depending on plant size using a drip irrigation system with half-strength nutrient solution as described in Robinson (1984). Soil moisture tension at 25 cm from the surface was monitored using tensiometers and was near 2 kPa throughout the experiment. Shade curtains to simulate border closure were not used in this experiment resulting in greater sunlit leaf area than in closed canopy studies (Kim et al., 2004). Photosynthetically active radiation (PAR) was measured with quantum sensors (LI-190SB; LI-191, LI-COR Inc., Lincoln,
Environmental conditions inside SPAR units during the growing season

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>C_a (μmol mol⁻¹)</th>
<th>PAR (μmol m⁻² s⁻¹)</th>
<th>Air T (°C)</th>
<th>Soil T (°C)</th>
<th>RH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient C_a</td>
<td>Dawn</td>
<td>563.7 ± 71.52</td>
<td>79.2 ± 7.74</td>
<td>29.6 ± 0.22</td>
<td>24.2 ± 0.47</td>
<td>70.9 ± 8.27</td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td>375.7 ± 5.32</td>
<td>716.4 ± 10.83</td>
<td>30.8 ± 0.19</td>
<td>26.0 ± 0.27</td>
<td>72.5 ± 7.11</td>
</tr>
<tr>
<td></td>
<td>Dusk</td>
<td>456.1 ± 26.12</td>
<td>92.3 ± 5.95</td>
<td>31.0 ± 0.24</td>
<td>26.2 ± 0.25</td>
<td>70.5 ± 7.31</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>580.3 ± 75.07</td>
<td>0.14 ± 0.020</td>
<td>25.6 ± 0.16</td>
<td>24.3 ± 0.41</td>
<td>79.1 ± 6.07</td>
</tr>
<tr>
<td>Elevated C_a</td>
<td>Dawn</td>
<td>747.8 ± 3.25</td>
<td>71.7 ± 1.72</td>
<td>29.6 ± 0.24</td>
<td>23.8 ± 0.10</td>
<td>60.0 ± 5.97</td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td>736.3 ± 2.05</td>
<td>696.7 ± 22.27</td>
<td>30.8 ± 0.12</td>
<td>25.3 ± 0.60</td>
<td>61.8 ± 7.97</td>
</tr>
<tr>
<td></td>
<td>Dusk</td>
<td>754.4 ± 1.71</td>
<td>97.1 ± 5.28</td>
<td>30.9 ± 0.14</td>
<td>25.4 ± 0.45</td>
<td>60.2 ± 6.42</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>747.9 ± 2.72</td>
<td>0.16 ± 0.031</td>
<td>25.8 ± 0.55</td>
<td>23.8 ± 0.30</td>
<td>69.3 ± 5.21</td>
</tr>
</tbody>
</table>

Time of day was grouped to dawn (4:00–8:00 hours), day (8:00–16:00 hours), dusk (16:00–20:00 hours), and night (0:00–4:00 and 20:00–24:00 hours). Shown are mean and standard deviations (n = 3) of CO₂ concentration (C_a), photosynthetically active radiation (PAR), air temperature (Air T), soil temperature averaged over four depths (Soil T), and relative humidity (RH).

SPAR, soil–plant–atmosphere research.

NE, USA)³ located at the top and bottom of the canopy, and outside the chambers. Soil temperatures were measured at four depths using thermocouples. Environmental conditions inside SPAR chambers are summarized in Table 1. Plants were harvested between 69 and 71 DAP when the plants were at developmental stage R3 (i.e. the milky ripe stage), according to the system developed by Ritchie et al. (1997). At the completion of the experiment, shoots were detached at ground level and the leaves, tillers, and ears were separated. Total laminar area per plant was measured with a leaf area meter (LI-3000, LI-COR Inc.). Harvested plant parts were dried in a forced-air oven at 70°C for at least 72 h prior to determining dry weight. Leaf ligule appearance was monitored on five plants per chamber four to six times per week to determine leaf developmental rates. Senescence was determined based on the number of dropped, withered, or brown leaves. Silking date was defined as the day that one-half of the plants had silk visible on the outside of the husks. Three fully expanded leaves near the top of the canopy were sampled from each chamber 45 DAP, and total carbon and nitrogen were analyzed by combustion (CHN-2000, LECO Corporation, St Joseph, MI, USA).

Canopy gas exchange measurements

The C_a of each chamber was measured continuously with a dedicated infrared gas analyzer (Model LI-6252, LI-COR Inc.). Chamber CO₂ was supplied as medical grade CO₂ from a compressed gas cylinder (Potomac Airgas Inc., Linthicum, MD, USA). Injection rates of CO₂ were regulated by mass flow controllers (FMA-766-V-CO₂, Omega Engineering Inc., Stanford, CT, USA) located in the air handling system of each chamber using a feed-forward, feed-back proportional–integral–differential (PID) control algorithm. Canopy CO₂ exchange rates (A_can) were calculated from mass balance equations, solved every 30 s, averaged, and recorded every 300 s. To correct A_can for chamber leakage, chamber CO₂ leakage rates were determined daily using a N₂O drawdown method (Baker et al., 2004). Canopy evapotranspiration rates (ET_can) were determined by collecting the condensate from cooling coils used to control air temperature and relative humidity and passing the condensate through a conduit connected to pressure transducers. Canopy temperature was measured using an infrared thermometer (Model 4000.4ZL, Everest Interscience Inc., Tucson, AZ, USA) located above the canopy in each chamber.

Leaf gas exchange and chlorophyll (Chl) fluorescence measurements

A portable photosynthesis system (LI-6400, LI-COR Inc.) with a red/blue LED light source (LI6400-02B) mounted onto a 6 cm² clamp-on leaf chamber was used to determine the responses of CO₂ assimilation to light (A/Q) and CO₂ (A/C). Two or three plants per chamber were selected, and recently developed fully expanded young leaves (11, 12, or 13th leaves of the main stem) were used for A/C, and A/Q response determinations on 35, 36, or 38 DAP between 09:00 and 16:00 hours. The A/C measurements were made at 10 CO₂ levels between 50 and 1200 μmol mol⁻¹ air with PAR inside the leaf cuvette of 2000 μmol m⁻² s⁻¹. The A/Q response was determined at nine PAR levels between 0 and 2500 μmol m⁻² s⁻¹ at respective growth C_a. Leaf temperature was kept at 31.1 ± 0.25°C and vapor pressure deficit was at 2.1 ± 0.30 kPa.

The photochemical efficiency of PSII was determined as the relative variable Chl fluorescence (F_v/F_m) using
a pulse amplitude modulated Chl fluorescence system (Model OS-500, Opti-Sciences Inc., Tyngsboro, MA, USA) about 1 h before dawn. The illumination and measurement signals were provided by a trifurcated light guide held at either 45° or 90° to the upper leaf surface. Red modulating light and saturating flashes of white light were from the fluorometer. Fluorescence measurements were performed daily using the most recent, fully expanded leaf of all plants in each chamber over a 10 day period beginning 25 DAP.

**Leaf pigments and enzyme assays**

Samples for determining Chl content and C₄ enzyme activities were collected 30, 35, and 42 DAP within 2 h of solar noon on the assigned dates. Four 1.65 cm² leaf discs were removed from the lamina of the most recent, fully expanded leaf of three plants from each chamber. The disks were rapidly transferred to labeled envelopes, immediately immersed in liquid N₂ to quench metabolism. All samples were stored for a maximum of 1 month at −80 °C prior to use. One leaf disc from each plant was extracted with 1 mL 80% acetone, and Chl a and b were quantified from optical density measurements according to Lichtenthaler (1987). The remaining three leaf discs from each plant were extracted with 1.5 mL ice cold extraction buffer consisting of 50 mM Tris-HCl (pH 7.50), 10 mM MgCl₂, 1 mM EDTA, 1% (w/v) PVP-40, 5 mM Na⁺-pyruvate, and 10% glycerol. Immediately prior to extraction the solution was made to 1 μM leupeptin and 5 μM dithiothreitol. Leaf material (4.95 cm²) was extracted at 0 °C with a ground glass tissue homogenizer and the homogenates were transferred to 2 mL plastic centrifuge tubes. The samples were spun in a centrifuge (Model 5415C, Brinkmann, Westbury, NY, USA) for 3 min at 14 000 × g and 0.23 mL aliquots of each supernatant was immediately transferred to four separate 0.5 mL centrifuge tubes on ice. The aliquots were quickly placed in liquid N₂ until used for analysis. Enzyme activities were determined spectrophotometrically at 25 °C as described by Maroco et al. (1999). Briefly, NADP-malate dehydrogenase (MDH) was measured in 1 mL solution containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM oxalacetic acid, 10 mM NADPH, and 0.025 mL leaf extract. Phosphoenol pyruvate carboxylase (PEPC) was measured in 1 mL solution containing 50 mM Tris-HCl (pH 8.0), 5 mM NaHCO₃, 5 mM MgCl₂, 10 mM NADH, 10 mM PEP (tricyclohexylamine salt), 1 U MDH, and 0.025 mL sample. NADP-malic enzyme (NADP-ME) was measured in 1 mL solution containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM NADH, 1 mM oxalacetic acid, 10 mM NADPH, and 0.025 mL sample. All measurements were performed using a spectrophotometer (Model 2101, Shimadzu Scientific Instruments, Columbia, MD, USA) operated in the kinetic mode. Enzyme activities were calculated from the rate of change in optical density at 340 nm. Enzyme activity and Chl content results were averaged across dates for analyses.

**Determination of C₄ photosynthesis model parameters and statistical analysis**

The biochemical model of C₄ photosynthesis by von Caemmerer & Furbank (1999) was fitted to the A/Cᵢ and A/Qᵢ response curves using SAS NLIN (SAS ver 8.12, SAS Institute Inc., Cary, NC, USA). The initial slope and rate saturated region of the A/Cᵢ curves were used to estimate carboxylation efficiency, maximum PEPC activity (Vᵢₘₐₓ), and maximum Rubisco activity (Vᵢₘₐₓ). The initial slope and light saturated asymptote values of A/Qᵢ curves were used to estimate apparent quantum efficiency and maximum electron transport rate (Jₑₘₐₓ). Other parameters were used as described by von Caemmerer & Furbank (1999).

Each chamber was an experimental unit and treated as a replication, such that n = 3. All measurements on different plants within each chamber were regarded as subsamples. Statistical differences in growth, leaf gas exchange rates, enzyme activities, and other characteristics of plants grown at ambient or elevated C₄ chambers were determined using a Student’s t-test method assuming equal variances using SAS TTEST. Daily canopy gas exchange rates and diurnal canopy temperatures were analyzed as repeated measures using SAS MIXED procedure as measurements were repeated over time for each chamber (Littell et al., 1996). Linear regressions of hourly mean of canopy minus air temperature (Tₑₐₑ) as a function of PAR in relation to CO₂ treatment were also analyzed using SAS MIXED.

**RNA extraction and hybridization**

Leaf tissue (~ 10 cm²) for RNA extraction and DNA microarray analysis was removed from the central portions (excluding the mid-rib) of the most recent, fully expanded leaves of two plants in each chamber during the middle of the photoperiod on 35 DAP. Samples from each chamber were rapidly transferred to labeled envelopes and immediately frozen in liquid N₂ to stop metabolism. The samples were then held under liquid N₂ until analysis. Prior to extraction, equal amounts of leaf material from two plants within the same chamber were combined. Frozen leaf material was ground to a powder with liquid N₂ in a mortar and pestle and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Maize DNA microarrays were obtained from the Maize Gene Discovery Project at
the University of Arizona (array type: Unigene 1.02.02, http://www.maizegdb.org/microarray.php). RNA extraction and other procedures were based on protocols recommended by the University of Arizona (http://www.maizegdb.org/documentation/mgdp/microarray/protocols.php). A total of 7442 expressed sequence tags (EST) were printed on each microarray and each spot was replicated at least once generating a total of 16,926 spots including blanks. Each slide was replicated and one replicated pair of microarray slides was used for each sample. Labeling of total RNA with 5'Cy3 or 5'Cy5 random 9-mers, hybridization of the labeled probes to the slides and imaging procedures were performed at the University of Maryland, DNA Microarray Core Facility located in College Park, Maryland, USA (http://www.umbi.umd.edu/~cbr/macore/macorestart.htm).

Microarray data analysis

Scanned images of microarray slides were analyzed using ScanAlyze software (version 2.50) from Stanford University (http://genome-www5.stanford.edu/resources/restech.shtml). Spots with high background were filtered and the data were normalized with a mixed linear model by Wolfinger et al. (2001) using the SAS MIXED procedure (Littell et al., 1996). This normalization procedure removed random variability because of chambers, slides, or spots and corrected for any detectable dye effects. The data were then transformed with a logarithm to the base 2 and the effects of elevated \( C_a \) treatment on gene expression were modeled and evaluated by determining the experimentwise error rate with the Bonferroni adjustment (Wolfinger et al., 2001). The critical \( P \)-value after the Bonferroni adjustment for pairwise comparison was 1.34e-6 (\( = 0.01/7442 \)). This fixed the experimentwise error rate at \( P = 0.01 \). To be conservative, only those genes with greater than twofold change and a \( P \)-value less than the critical value were marked significant and selected for functional classification. All significantly induced or repressed genes with known functions were classified into groups based on gene ontology (GO) information obtained from the TIGR Maize Gene Index Database (http://www.tigr.org/tdb/zmgi/). GenBank accession numbers and homologue information corresponding to the microarrays used in this study were obtained from Maize Genetics and Genomics Database (http://www.maizegdb.org/).

### Results

#### Biomass, growth, and development

Dry matter allocated to leaves, stems, tillers, and ears did not differ between treatments (Table 2). Total shoot dry mass was 220 and 227 g plant\(^{-1}\) (\( P = 0.73 \)) for plants grown in the ambient and elevated \( C_a \) treatments, respectively. Leaf area per plant and specific leaf area (SLA) also did not respond to elevated \( C_a \). The number of tillers per plant was 1.52 and 2.06 (\( P = 0.08 \)) in the ambient and elevated \( C_a \) treatments, respectively. Days to silking did not differ between the \( \mathrm{CO}_2 \) treatments (\( P = 0.39 \)). Rates of ligule appearance and the development of senescent leaves also were similar between treatments (data not shown).

#### Canopy gas exchange rates and canopy temperatures

Diurnal patterns of canopy gas exchange rates are shown 21, 32, and 39 DAP when plants were at the V6, V10, and V14 growth stages, respectively (Fig. 1). At these growth stages, \( \mathrm{CO}_2 \) enrichment did not enhance canopy photosynthesis (\( P = 0.62, 0.32, \) and 0.46, respectively). Plants grown at elevated \( C_a \) consistently exhibited lower canopy evapotranspiration rates than those

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**Table 2** Biomass and other growth parameters of maize plants grown under ambient and elevated \( C_a \)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Ambient ( C_a )</th>
<th>Elevated ( C_a )</th>
<th>( \text{SE}_{\text{diff}} )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Above ground biomass</td>
<td>g plant(^{-1})</td>
<td>219.6</td>
<td>226.7</td>
<td>18.7</td>
<td>0.726</td>
</tr>
<tr>
<td>Stem biomass</td>
<td>g plant(^{-1})</td>
<td>57.7</td>
<td>64.2</td>
<td>4.1</td>
<td>0.191</td>
</tr>
<tr>
<td>Leaf biomass</td>
<td>g plant(^{-1})</td>
<td>20.4</td>
<td>20.5</td>
<td>1.4</td>
<td>0.931</td>
</tr>
<tr>
<td>Ear biomass</td>
<td>g plant(^{-1})</td>
<td>60.6</td>
<td>51.4</td>
<td>5.4</td>
<td>0.164</td>
</tr>
<tr>
<td>Tiller biomass</td>
<td>g plant(^{-1})</td>
<td>81.0</td>
<td>90.5</td>
<td>11.0</td>
<td>0.436</td>
</tr>
<tr>
<td>Tillers</td>
<td>ea</td>
<td>1.52</td>
<td>2.06</td>
<td>0.23</td>
<td>0.080</td>
</tr>
<tr>
<td>Leaf area</td>
<td>m(^2) plant(^{-1})</td>
<td>0.760</td>
<td>0.878</td>
<td>0.071</td>
<td>0.183</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>cm(^2) g(^{-1})</td>
<td>193.7</td>
<td>199.1</td>
<td>7.2</td>
<td>0.495</td>
</tr>
<tr>
<td>Days to silking</td>
<td>days</td>
<td>45.0</td>
<td>46.4</td>
<td>1.02</td>
<td>0.387</td>
</tr>
</tbody>
</table>

\( \text{SE}_{\text{diff}} \) represents one standard error of the difference between the treatments. \( P \) is the probability that the difference between treatments is not significantly different from zero.
grown at ambient $C_a$ across the three growth stages ($P = 0.05, 0.03,$ and $0.06$, respectively). Diurnal canopy CO$_2$ uptake (6:00–18:00 hours) was similar between treatments (Fig. 2a; $P = 0.37$) whereas diurnal canopy evapotranspiration rates in elevated $C_a$ treatment were reduced by 21.5% compared with the values in ambient $C_a$ (Fig. 2b; $P = 0.03$) from emergence to silking. Canopy temperatures were usually greater in the elevated compared with the ambient $C_a$ treatments during the day (Fig. 3a). Canopy temperature minus air temperature ($T_{\text{diff}}$) between 10:00 and 15:00 hours was increased by 0.92 $^\circ$C in response to CO$_2$ enrichment ($P = 0.09$). $T_{\text{diff}}$ was similar between treatments when PAR is low (i.e. dawn, dusk, and night) (Fig. 3b inset). $T_{\text{diff}}$ was positively correlated to PAR near noon while relatively insensitive to PAR during dawn and dusk in both treatments (Fig. 3b). The slopes for linear regression of $T_{\text{diff}}$ on PAR both near noon and during dawn–dusk were heterogeneous between ambient and elevated $C_a$ ($P < 0.05$).

Leaf gas exchange rates and Chl fluorescence

At saturating PAR (i.e. 2000 $\mu$mol m$^{-2}$ s$^{-1}$) and at growth $C_a$, $A_{\text{leaf}}$ of plants grown at ambient and elevated $C_a$ were 48.7 and 50.6 $\mu$mol m$^{-2}$ s$^{-1}$, respectively ($P = 0.41$; Table 3). Leaves grown at elevated $C_a$ exhibited over 50% reduction in stomatal conductance ($g_s$) and transpiration ($E_{\text{leaf}}$) (Table 3). Intercellular CO$_2$ concentration ($C_i$) values were 169 and 312 $\mu$mol mol$^{-1}$ ($P = 0.003$) for leaves grown under ambient and elevated $C_a$, respectively. This resulted in similar $C_i/C_a$
ratios of 0.46 and 0.42 for the ambient and elevated $C_a$ treatments, respectively ($P<0.44$). As a result of reduced $g_s$ and $E_{leaf}$, instantaneous WUE measured as $A_{leaf}/E_{leaf}$ was greater for leaves of plants grown under elevated compared to ambient $C_a$ ($P<0.001$).

Light response curves measured at growth $C_a$ were similar for leaves grown at either $C_a$ treatment (Fig. 4a). However, the $A/C_i$ response curves revealed that $A_{leaf}$ determined at low $C_i$ generally was lower for plants grown at elevated $C_a$ compared with ambient $C_a$ (Fig. 4b). Conversely, $A_{leaf}$ measured at high $C_i$ was similar between treatments (Fig. 4b). The carboxylation efficiency estimated as the initial slope of $A/C_i$ response was lower ($P<0.04$) under elevated $C_a$ than under ambient $C_a$ (Table 3). Estimates of $V_{p_{\text{max}}}$ of the $C_4$ photosynthesis model were 120.4 and 91.9 mol m$^{-2}$s$^{-1}$ for ambient and elevated $C_a$ ($P=0.05$), respectively. Estimates of $V_{c_{\text{max}}}$ and $J_{\text{max}}$ were similar between the treatments. There were no differences in $F_v/F_m$ between ambient and elevated $C_a$ (Table 3).

Leaf constituents and enzyme activities

The activities of three key enzymes of the $C_4$ cycle: PEPC, MDH, and NADP-ME were similar for leaves of ambient and elevated $C_a$ grown plants (Table 3). Leaf concentrations of Chl a, Chl b, and total Chl ($a+b$) did not differ for plants grown under ambient and elevated $C_a$. However, the ratio of Chl a/b was lower under elevated compared with ambient $C_a$ ($P=0.08$; Table 3). Total carbon was approximately 45% of the total dry mass for leaves from the ambient and elevated $C_a$ treatments. Leaf tissue contained 3.0% and 3.3% N (w/w) ($P=0.04$) for plants grown in the elevated compared with the ambient $C_a$ treatments, respectively. Consequently, the leaf C/N ratio was 14.8 under elevated $C_a$ and this compared with a value of 13.7 under ambient $C_a$ ($P=0.02$).
Table 3  C₄ cycle enzyme activities, chlorophyll content, leaf nitrogen and carbon content, leaf gas exchange rates at PAR = 2000 μmol m⁻² s⁻¹, and C₄ photosynthesis model parameters of maize plants grown under ambient and elevated C₄

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Ambient C₄</th>
<th>Elevated C₄</th>
<th>SEdiff</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical properties</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEPC</td>
<td>μmol m⁻² s⁻¹</td>
<td>43.54</td>
<td>42.32</td>
<td>2.72</td>
<td>0.676</td>
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<tr>
<td>MDH</td>
<td>μmol m⁻² s⁻¹</td>
<td>23.99</td>
<td>24.01</td>
<td>2.26</td>
<td>0.993</td>
</tr>
<tr>
<td>NADP-ME</td>
<td>μmol m⁻² s⁻¹</td>
<td>39.51</td>
<td>39.44</td>
<td>1.96</td>
<td>0.972</td>
</tr>
<tr>
<td>Chl a</td>
<td>g m⁻²</td>
<td>0.244</td>
<td>0.230</td>
<td>0.010</td>
<td>0.253</td>
</tr>
<tr>
<td>Chl b</td>
<td>g m⁻²</td>
<td>0.053</td>
<td>0.052</td>
<td>0.002</td>
<td>0.528</td>
</tr>
<tr>
<td>Leaf total carbon</td>
<td>% in w/w</td>
<td>45.07</td>
<td>44.67</td>
<td>0.063</td>
<td>0.084</td>
</tr>
<tr>
<td>Leaf total nitrogen</td>
<td>% in w/w</td>
<td>3.29</td>
<td>3.03</td>
<td>0.089</td>
<td>0.040</td>
</tr>
<tr>
<td>Leaf C/N ratio</td>
<td></td>
<td>13.71</td>
<td>14.81</td>
<td>0.311</td>
<td>0.024</td>
</tr>
<tr>
<td>Leaf gas-exchange rates and chlorophyll fluorescence</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A_leaf</td>
<td>μmol m⁻² s⁻¹</td>
<td>48.72</td>
<td>50.63</td>
<td>2.07</td>
<td>0.409</td>
</tr>
<tr>
<td>gₛ</td>
<td>mol m⁻² s⁻¹</td>
<td>0.525</td>
<td>0.207</td>
<td>0.079</td>
<td>0.016</td>
</tr>
<tr>
<td>I_leaf</td>
<td>mmol m⁻² s⁻¹</td>
<td>8.61</td>
<td>4.05</td>
<td>0.78</td>
<td>0.004</td>
</tr>
<tr>
<td>Cₛ</td>
<td>μmol mol⁻¹</td>
<td>168.5</td>
<td>312.0</td>
<td>21.98</td>
<td>0.003</td>
</tr>
<tr>
<td>Cₛ/C₄</td>
<td>–</td>
<td>0.455</td>
<td>0.417</td>
<td>0.044</td>
<td>0.437</td>
</tr>
<tr>
<td>WUE</td>
<td>μmol (CO₂)/mmol (H₂O)</td>
<td>6.05</td>
<td>12.58</td>
<td>0.086</td>
<td>0.001</td>
</tr>
<tr>
<td>Fᵣ/Fₘ</td>
<td>–</td>
<td>0.8104</td>
<td>0.8097</td>
<td>0.0016</td>
<td>0.710</td>
</tr>
<tr>
<td>C₄ photosynthesis model parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantum efficiency</td>
<td>mol(CO₂)/mol (quanta)</td>
<td>0.073</td>
<td>0.071</td>
<td>0.011</td>
<td>0.868</td>
</tr>
<tr>
<td>Carboxylation efficiency</td>
<td>(μmol m⁻² s⁻¹)/ (μmol mol⁻¹)</td>
<td>0.804</td>
<td>0.610</td>
<td>0.064</td>
<td>0.038</td>
</tr>
<tr>
<td>Vₚmax</td>
<td>μmol m⁻² s⁻¹</td>
<td>120.4</td>
<td>91.9</td>
<td>10.50</td>
<td>0.054</td>
</tr>
<tr>
<td>Vₚmax</td>
<td>μmol m⁻² s⁻¹</td>
<td>67.3</td>
<td>71.6</td>
<td>4.03</td>
<td>0.347</td>
</tr>
<tr>
<td>fₚmax</td>
<td>μmol m⁻² s⁻¹</td>
<td>349.2</td>
<td>354.2</td>
<td>18.2</td>
<td>0.796</td>
</tr>
</tbody>
</table>

SEdiff represents one standard error of the difference between treatments. P is the probability that the difference is not significantly different from zero. C₄ photosynthesis model parameters were estimated by fitting the initial slope and saturated rates of the A/C₄ and A/Q curves for each treatment using the photosynthesis model described by von Caemmerer & Furbank (1999).

DNA microarray analysis

Out of 7442 ESTs printed on each slide there were a total of 387 transcripts that were differentially expressed in the ambient and elevated C₄ treatments. A significance plot displaying the relationship between the log₂ of the estimated expression change and the – log₁₀ of P-values for each transcript is shown in Supp. Fig. S1. There were 227 and 160 maize transcripts that were induced or repressed, respectively, in response to CO₂ enrichment. It should be noted that the maize genome has not been completely sequenced or annotated. As a result, 279 out of the 387 transcripts identified in this experiment were unclassified and had no known GO. The remaining 108 transcripts were classified into functional groups (Fig. 5). Of these only 15 transcripts have been completely sequenced and have known functions to date. A list of genes altered by CO₂ enrichment is supplemented in Table S1.

A total of 51 maize transcripts were induced by CO₂ enrichment that also had some degree of annotation in the Maize Genome Database. Notably, two induced transcripts, AW011661 and AL657231, were putative calcium dependent protein kinases; calcium, or calmodulin regulated protein kinases that are linked to salinity or drought stress (Patharkar & Cushman, 2000). Other potentially important induced transcripts included AI670650 encoding S-adensylmethionine decarboxylase proenzyme (AdoMetDC), AI665712 encoding Brittle-1, and AI586416 encoding ferredoxin. AdoMetDC is a key regulatory enzyme in polyamine biosynthesis (Hanfrey et al., 2003). Brittle-1 functions as a proposed adenylate translocator in maize amyloplasts (Shannon et al., 1998). Ferredoxins are crucial, soluble, low molecular weight, iron-sulfur proteins that function as electron carriers in various redox reactions (Hirohashi et al., 2001).

There were 57 annotated maize transcripts that were repressed by CO₂ enrichment. Notably, several putative and known genes involved in photosynthesis or in light capture in the chloroplast were repressed. These included AI947633, AI649549, two transcripts encoding a photosystem-II 10K protein [Oryza sativa], AL600810, a photosystem-I assembly protein ycf3 (IRF170) [Zea
and a Chl a/b-binding protein (CP26) precursor [Zea mays]. Also repressed were AW331243 and AW438072, two transcripts for the precursor of the Rubisco small subunit protein [Zea mays]; AI737746 and AI881961, encoding the cytosolic isoform of glyceraldehyde 3-phosphate dehydrogenase (G3PDH); and AI739968 and AI603703, encoding fructose-1,6-bisphosphate aldolase and a putative fructose-1,6-bisphosphatase, respectively. A potentially important repressed transcript was AI676939 that encodes leucine-rich repeat (LRR) protein (Table 4). LRR protein was shown to be closely related to control of stomatal distribution in Arabidopsis (Nadeau & Sack, 2002). Other potentially important maize genes that were repressed by CO2 enrichment included AI770450, a major intrinsic protein, AI677017, a cyclophilin, and AI674006 a high mobility group protein-d1 (HMGd1). Major intrinsic proteins are a family of periplasmic membrane proteins located on the plasma membrane and tonoplast that includes water channel proteins (Chaumont et al., 2000). Cyclophilins, also known as rotamases or peptidyl-prolyl cis-trans isomerases, are present throughout the plant and are involved in protein folding (Romano et al., 2004). Cyclophilins also may function in numerous important cellular processes including mRNA processing, protein degradation and in signal transduction. HMG proteins are thought to function in chromatin architecture (Renner et al., 2000).

Discussion

The response of plants possessing the C4 photosynthetic pathway to elevated Ca is complicated by the presence of a CO2 concentrating mechanism and to date results have been variable and difficult to explain. Although several previous studies reported that CO2 enrichment enhanced the growth of maize under well watered and fertilized conditions, in the current study there was little evidence of increased biomass accumulation. This was further supported by a lack of response in leaf and canopy photosynthetic rates to the elevated Ca. Elevated Ca may increase the growth of maize via increases in Ci or by changes in diurnal CO2 fixation patterns. Our results indicate that although Ci was increased neither Aleaf nor Acan responded to the elevated Ca. Diurnal canopy CO2 fixation patterns also were similar between ambient and elevated Ca regardless of growth stages (Fig. 1a). Reduced C4 cycle capacity normally results in a decrease in the initial slope of A/Ci curve and an increase in the CO2 saturation point if saturating substrate levels are assumed (Sage, 1994; von Caemmerer & Furbank, 1999). We observed a decrease of carboxylation efficiency in the
Table 4  Expression changes of maize transcripts that are potentially linked to stomatal development and distribution in response to CO2 enrichment

<table>
<thead>
<tr>
<th>Accession no</th>
<th>Description</th>
<th>Fold change</th>
<th>P</th>
<th>Gene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI676939</td>
<td>Leucine-rich repeat protein</td>
<td>0.46</td>
<td>0.005</td>
<td>TMM</td>
</tr>
<tr>
<td>AI622721</td>
<td>Putative beta-ketoacyl-CoA synthase</td>
<td>0.54</td>
<td>0.017</td>
<td>HIC</td>
</tr>
<tr>
<td>AI737399</td>
<td>Putative subtilisin serine protease</td>
<td>0.31</td>
<td>0.098</td>
<td>SDDI</td>
</tr>
<tr>
<td>AI978071</td>
<td>Mitogen-activated protein kinase kinase</td>
<td>1.71</td>
<td>0.011</td>
<td>YODA</td>
</tr>
</tbody>
</table>

Description of potential gene products was obtained from the MaizeGDB website (http://www.maizegdb.org/). P is the probability representing the experimentwise error rate. Fold expression changes were expressed as the ratio of elevated C4/ambient C4.

*The Arabidopsis genes known to be involved in stomatal development with corresponding function.

...plants grown at elevated C4 (see also Maroco et al., 1999; Watling et al., 2000). However, this decreased carboxylation efficiency was not accompanied by decreased PEPC activity in vitro or repression of the transcripts encoding PEPC. Reasons for this disparity were not obvious. On the other hand, elevated C4 significantly reduced water loss at the single leaf and canopy levels. Moreover, instantaneous leaf transpiration rate at high PAR was reduced over 50% and cumulative canopy evapotranspiration rates were decreased by 22% under elevated C4. Consequently, reduced ETc at elevated C4 resulted in increased canopy WUE (P = 0.07). The difference between leaf and canopy relative reductions in evapotranspiration probably resulted from the fact that a portion of leaf area was shaded in the canopy because the difference in gs between ambient and elevated C4 would diminish as PAR decreases. This reduction in ETc is comparable to findings from other studies of C4 plants (Samarakoon & Gifford, 1996; Conley et al., 2001; Triggs et al., 2004). It should be noted that physiological responses of C4 plants to high C4 may depend on species or C4 subgroups so that a caution should be given when comparing results between species.

Measured leaf temperatures at the top of the canopy were greater in the elevated than in ambient C4 treatment probably because evapotranspiration rates and gs were decreased by CO2 enrichment. A similar increase in canopy temperature minus air temperature (Tcal) was observed previously in two C4 species exposed to elevated C4 and this increase was attributed to reduced gs (Siebeke et al., 2002). In the present study Tcal was positively correlated with PAR and was consistently greater at elevated C4 when PAR was relatively high (> 100 μmol m⁻² s⁻¹) (Fig. 3b). It is also suggested that leaf warming because of increasing PAR may be greater under elevated C4 compared with ambient C4 as the slope of the regression between PAR and Tcal was higher at high C4. In general C4 plants have higher optimal temperatures for photosynthesis and for growth in comparison with C3 plants (Long, 1999).

The lack of an effect of increased leaf temperature on Acan in the present study was probably because plants were grown near the temperature optimum for maize photosynthesis (Tollenar, 1989).

The DNA microarray analyses performed here identified 387 out of a total 7442 genes (5.2%) that displayed statistically significant, twofold or greater expression changes in response to elevated C4. Approximately 59% of these altered transcripts were induced by CO2 enrichment. Note that 73% of the maize transcripts that responded to elevated C4 lacked annotation. This is a common problem in species with an incompletely sequenced genome. In the present study, transcript levels were altered by elevated C4 without concomitant changes in the rates of photosynthesis, C4 cycle enzyme activities, growth, or development. Although leaf temperature was greater at elevated than at ambient C4, less than a 1°C difference in temperature near the growth temperature optimum was unlikely to have caused the large changes in transcript expression levels observed here. Therefore, it is difficult to fully explain the mechanisms underlying the observed changes in transcription profiles in response to CO2 enrichment. One possible mechanism to account for the large number of altered transcript levels in the present study can be linked to decreased leaf nitrogen level. Inorganic nitrogen and cellular constituents containing organic nitrogen are among the powerful signaling metabolites in plant cells. Nitrogen and carbon metabolism are carefully integrated processes that are coordinated at the level of transcription (Stitt & Krapp, 1999). Many of the effects of elevated C4 on nitrate metabolism, photosynthetic allocation, photosynthetic acclimation, and growth were due to a shift in N status in tobacco plants (Geiger et al., 1999). Total nitrogen content in young leaves was about 9% lower in plants grown under elevated compared to ambient C4 although it was not at deficient level in either treatment in the present study. Nitrate flux from the soil to the shoot was controlled by both transpiration rate and the delivery rate of nitrate to the transpiration stream by the roots in maize (Shaner & Boyer, 1976). Hence, the reduced transpiration rates at...
elevated C_a may lead to reduced nitrogen flux from the soil to the shoots (Conroy & Hocking, 1993; McDonald et al., 2002). This could potentially decrease the rates of N assimilation and partly account for the lower nitrogen content in the leaves grown in elevated C_a. The mechanism by which leaf nitrogen was decreased in the present study remains uncertain. Whether the reduction in leaf nitrogen was due to reduced nitrogen flux tied to reduced water uptake or was a result of acclimation which lowered nitrogen demand merits further investigation. In either case, the changes in nitrogen metabolism might have triggered signaling cascades that resulted in altered transcription profiles without notable changes in photosynthesis or growth. Hence, altered transcription profiles in CO_2 enriched maize plants could be a response to altered nitrogen metabolism in comparison to plants from the ambient C_a.

Results for several transcripts that responded to the elevated C_a were in agreement with prior findings. Two repressed maize transcripts (AW331243; AW438072) were orthologous to Rubisco small subunit genes (rbcS) from other species. Rubisco activity was reduced in previous maize studies using elevated C_a treatments (Wong, 1979; Maroco et al., 1999) and Rubisco protein and mRNA encoding both Rubisco small and large subunits declined significantly in response to long-term exposure to elevated C_a in Arabidopsis (Cheng et al., 1998). Two other related transcripts that were repressed in this study were AI739968 and AI603703. These encoded maize fructose-1,6-bisphosphate aldolase and a putative fructose-1,6-bisphosphatase, respectively. Two transcripts that encoded cytosolic G3PDH in maize (AI737746; AI881961) also were down-regulated in this study. These findings suggested that the genes encoding the key maize enzymes involved in glycolytic metabolism were repressed by the elevated C_a treatment. The activity of maize G3PDH also was decreased in response to elevated C_a in a prior study (Maroco et al., 1999). This merits further investigation as the effects of elevated C_a on inhibiting dark respiration rate per dry weight is still in debate (Drake et al., 1999; Bunce 2004; Davey et al., 2004; Gonzalez-Meler et al., 2004).

Stomatal development is controlled by C_a during early leaf development (Gray et al., 2000; Lake et al., 2001). A potentially important transcript (AI676939) encoding LRR protein which might be closely linked to stomatal development was altered by high C_a in the present study (Table 4; Table S1). In Arabidopsis, LRR protein is encoded by the gene TOO MANY MOUTHIS (TMM) which is involved in the regulation of stomatal initiation and distribution (Nadeau & Sack, 2002). LRR-kinase may act as a receptor for signals related to stomatal patterning but its mechanism has yet to be identified (Fleming, 2005). Interestingly, another potentially related transcript (AW120176) encoding LRR transmembrane protein kinase was induced in response to high C_a (Table S1). Other genes that are known to be involved in stomatal development include HIC (Gray et al., 2000) and SDD1 (Berger & Altmann, 2000). HIC encodes a putative 3-keto acyl coenzyme A synthase involved in the synthesis of very-long-chain fatty acids and acts as a regulator of stomatal development in response to C_a (Gray et al., 2000). A potentially related transcript (AI622721) encoding putative β-ketoacyl coenzyme A synthase was marginally down-regulated in response to high C_a (Table 4). This also suggests possible negative regulation of stomatal development. SDD1 encodes a subtilisin-like serine protease and is involved in signal processing that leads to guard cell formation (Berger & Altmann, 2000). Partially similar to the gene encoding subtilisin-like serine protease was AI737399 which was marginally repressed in the present study. These three genes (i.e. TMM, HIC, and SDD1) were down-regulated in leaves that lacked guard cells as a result of a constitutive activation of YODA in Arabidopsis (Bergmann et al., 2004). YODA encodes a mitogen-activated protein kinase kinase (MAPKK) kinase, which functions in determining guard cell identity and formation (Bergmann et al., 2004). In the present study, all three transcripts, AI676939, AI622721, and AI737399, exhibiting functional similarities to TMM, HIC, and SDD1 were down-regulated by elevated C_a (Table 4). In addition, the transcript (AI978071) encoding MAPKK was up-regulated indicating potential changes in guard cell formation in response to high C_a (Table 4). CO_2 enrichment reduced both stomatal opening and density in Arabidopsis (Lake et al., 2001) and maize (Maroco et al., 1999). The above findings suggest that, in agreement with the findings in Arabidopsis, the genes involved in stomatal development in C_a plants may be altered under elevated C_a and that responses to high CO_2 in C_a plants may be channeled by sensing of CO_2 in regulation of stomatal development and distribution.

In conclusion, elevated C_a did not enhance growth or A_can of well-watered and fertilized maize plants. Stomatal conductance and transpiration rates, both on a single leaf and on a canopy basis, were decreased in response to elevated C_a, thereby indicating increased WUE. Leaf temperature was increased and nitrogen content in young sunlit leaves was decreased at elevated C_a. The above findings suggested that most physiological responses to elevated C_a in maize may stem from altered water balance because of lowered stomatal conductance. This was further supported by down-regulation of the genes potentially linked to stomatal development at high C_a. Although more than 5% of the maize transcripts tested by DNA microarray...
techniques were significantly altered in response to elevated C\textsubscript{a}, it was difficult to fully associate the changes in transcription profiles with physiological responses partly because many of the altered genes had unknown function or were poorly annotated to date. While the responses of C\textsubscript{4} plants to elevated C\textsubscript{a} may be species specific, the list of those transcripts that were responsive to elevated C\textsubscript{a} in this study may serve as a useful resource for studying molecular mechanisms of physiological responses to elevated C\textsubscript{a} in C\textsubscript{4} plants. Future studies may investigate the protein expression levels using such techniques as two-dimensional gel electrophoresis to determine the relationships among protein levels, transcripts profiles, and metabolic changes in response to CO\textsubscript{2} enrichment.

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Supplementary material

The following material is available for this article online: http://www.ars.usda.gov/sp2UserFiles/Place/12755100/KimSH_GCB(2006)_Supplementary.pdf

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