INTERACTIVE EFFECTS OF ELEVATED CARBON DIOXIDE CONCENTRATION WITH NUTRIENT AVAILABILITY AND LEAF DEVELOPMENT ON PLANT CARBON METABOLISM

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Plant Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

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ABSTRACT

The balance between photosynthetic carbon dioxide (CO_2) assimilation and respiratory CO_2 release influence plant growth, crop yields, and the ability of terrestrial ecosystems to offset ~2-3 Gt CO_2 yr^{-1} of anthropogenic emissions. Rising atmospheric CO_2 concentration ($[CO_2]$) this century will impact plant photosynthesis and respiration with consequences for plant productivity in natural and agro-ecosystems. The capacity of all plants to grow and ecosystems to store carbon in elevated $[CO_2]$ can be dependent on interactions with water, nutrients, and plant developmental processes. The purpose of this thesis is to address fundamental knowledge gaps in understanding plant responses to the interaction between elevated $[CO_2]$ with water, nitrogen (N), and leaf developmental programs: (1) determine what is the mechanistic response of maize C_4 photosynthesis to a three way interaction between atmospheric $[CO_2]$, N availability and drought utilizing the unique capabilities of a Free Air CO_2 Enrichment (FACE) field experiment; (2) determine the transcriptional reprogramming of leaf respiration in response to growth in elevated $[CO_2]$ and variable N supply using *Arabidopsis thaliana* and a custom built gas exchange system; (3) determine when in leaf development the transcriptional reprogramming of respiration occurs in response to elevated $[CO_2]$ by studying the detailed developmental timelines and molecular events of leaf growth in *A. thaliana*. The knowledge gaps addressed in this work will help inform crop improvement and models that predict future ecosystem function and global food supply in the face of a changing climate.

ACKNOWLEDGEMENTS

I am grateful to my advisor Dr. Andrew Leakey for his friendship, excellent mentorship, always pushing me to achieve an extra 10%, and for enough intellectual freedom to work on some of my own ideas even if they turned out to be mostly un-fruitful. I would also like to thank the rest of my dissertation committee: Drs. Don Ort, Steve Long, and Steve Clough for helpful conversations and nuggets of wisdom that could only come out of many years of collective and diverse scientific experiences. I am very lucky to have Sharon Gray as a best friend, lab mate, and life partner that is (usually) willing to entertain any scientific idea I come across. I also feel very fortunate to have been a part of GEGC, PBAGS, and the Leakey lab that all serve as a large social network of fun and interesting people that I get to interact with on a daily basis, pose endless questions towards, and provide helping hands for conducting research. Of this large group of great folks, I am especially thankful for strong, lasting friendships formed with Paul Nabity, David Rosenthal, Sarah Davis and Chris Black. I am obliged to Lisa Ainsworth for her advice, insights, and the use of lab space and materials. People who have significantly contributed to help with protocols (thank you!) are Craig Yendrek, Kat Grennan, Bob Koester, Courtney Leisner, Amy Betzelberger, Kristen Bishop, Kannan Puthuval, Mike Masters, Shiv (there is only one), and Glenn Fried. Although I have had many great instructors while in graduate school, I would like to thank Dr. Tom Jacobs for really opening the world of plant development and molecular Arabidopsis research to me through an excellent course and subsequent stimulating conversations. I am very thankful for Melinda Laborg, Lisa Boise, Martha Plummer, Debbie Black, Lewis Sterling, Martha Lillard, and Nevo Band for their excellent professional support making my day-to-day run smoothly. I am indebted to the NSF for three years of graduate fellowship support. Finally, I would like to thank my mom, dad, brother, and the rest of my family for their love, support, understanding, and inspiration during my entire life.

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CHAPTER I: GENERAL INTRODUCTION

Since the beginning of the industrial revolution human activity has been significantly altering the global carbon cycle largely through the burning of fossil fuels and altering natural landscapes for agricultural production to support a growing human population (Canadell et al. 2007) and economic activity. As a consequence, the atmospheric carbon dioxide concentration ($[CO_2]$) has gradually risen from 280 ppm to over 390 ppm today and is projected to increase to over 700 ppm by the end of the century (Prentice et al. 2001). These levels of atmospheric $[CO_2]$ are greater than the last 20 million years (Pagani et al 2009; Pearson and Palmer 2000). Elevated atmospheric $[CO_2]$ is both a driver of anthropogenic global environmental change as a greenhouse gas and a factor contributing to changes in plant productivity due to greater substrate availability for photosynthetic carbon reduction in C_3 plants (Prentice et al. 2001; Ainsworth and Long 2005). How plants assimilate CO_2 and utilize the resulting chemical energy is a fundamental component of plant growth. The past twenty years of research has demonstrated enhanced leaf carbon gain in elevated $[CO_2]$ across many important agricultural plants including tobacco, wheat, rice, soybean, poplar, and perennial rye grass leading to significant stimulations in end of season yields (Kim et al. 1995; Kimball et al. 1995; Kruse et al. 1995; Adam et al. 2000; Taylor et al. 2001; Ainsworth et al. 2003; Morgan et al. 2005; Ainsworth and Long 2005). The literature as a whole clearly demonstrates that, in addition to variation among the major photosynthetic types (C_3, C_4, and CAM), the effects of elevated $[CO_2]$ on plant carbon balance vary depending on interacting genetic, environmental, and developmental factors and among plant functional groups (Sage and Kubien 2003; Zavaleta et al. 2003; Luo et al. 2008; Ghannom 2009; Kardol et al. 2010; Zhao et al. 2010; Albert et al. 2011). Understanding this variability is one of the major challenges in this field of research today. For specific examples, many uncertainties exist in how plants respond to elevated $[CO_2]$ when interactions with nutrient availability (Soussana et al. 2010), drought (Gerten et al. 2004; Ghannoum 2009), developmental time courses (Pritchard et al. 1999) or plant functional groups are considered (Leakey et al. 2009a; Gonzalez-Meler et al. 2004). Determining how elevated $[CO_2]$ interacts with environmental and genetic factors to alter the carbon (C) balance of plants is a crucial component for accurate prediction of future food supply, ecosystem function and fully adapting crops to exploit this additional atmospheric resource (Ainsworth et al. 2008; Leakey et al. 2009; Leakey et al. 2012). The open questions differ depending on if the plant functional group is C_3 or C_4.

Therefore this thesis research addresses three open questions relating to the interactive effects among elevated [CO_2] with drought, nitrogen availability, and a leaf developmental time course on leaf photosynthesis and respiration.

The C_4 plant functional type contributes 18% of global primary productivity and makes up about 40% of world grain harvest (Ehleringer et al. 1997; Patterson 1995; USDA-FAS 2005). Theoretically, under favorable growth conditions, C_4 photosynthesis is not stimulated by elevated [CO_2] (Ghannoum 2009). However, C_4 plants in natural and agricultural ecosystems frequently grow in conditions of limiting water availability and/or limiting N supply. These conditions may allow for the indirect enhancement of C_4 photosynthesis by elevated [CO_2] (Leakey 2009; Ghannoum and Conroy 1998). This is an important interaction to understand because summer precipitation events in mid-continental areas are projected to decrease in volume and frequency (Giorgi et al. 2001, Kling et al. 2003, Weltzin et al. 2003), and greater temperatures across the world will increase crop water use and deplete soil moisture, resulting in a greater risk of droughts this century (Meehl et al. 2007). Furthermore, areas where impacts of global climate change are projected to be the worst overlap significantly with regions in developing countries in Africa and Latin America that rely heavily on C_4 crops for food and where access to fertilizer is low (e.g. only 3% of world total fertilizer use in all of Africa; FAO 2008). Despite the indications that water and N availability mediate the effects of elevated [CO_2] on photosynthetic rates of C_4 plants (Ghannoum and Conroy 1998), few studies have examined the interaction among elevated [CO_2], water, and N availability on C_4 photosynthesis and yield in an open air field context that allows unrestricted rooting volumes and minimal disturbance on the soil-plant-atmosphere continuum. Globally important C_4 crops such as maize, sorghum and millet have rooting depths that reach 1-2 meters (Allen et al. 1998; Carcova et al. 2000) thereby greatly exceeding the soil volume (3-5 l) used in many pot studies (3-5 l; Wong 1979, Ziska and Bunce 1997, Maroco et al. 1999, Ziska et al. 1999) and calling into question if enough water could be supplied to the shoot even if small pots are well watered (Leakey 2009). All of these considerations are important because current models of future food supplies predict that C_4 photosynthesis and yield will be consistently enhanced by elevated [CO_2] and are parameterized by values that have not been validated in a field context (see details in Tubiello et al., 2007a, b).

Chapter 2 utilized the unique capabilities of a Free Air CO_2 Enrichment (FACE) field

experiment to test the interactive effects among atmospheric [CO_2], N availability and drought on C_4 maize photosynthesis and yield.

C_3 plants have a direct stimulation of photosynthesis under elevated [CO_2], which has been studied in many species (Ainsworth and Long 2005). N availability modulates the magnitude of the photosynthetic stimulation response to elevated [CO_2] and has been extensively studied across many species (Reviewed Stitt and Krapp 1999). In general, greater N availability leads to a greater stimulation in photosynthesis and productivity in elevated [CO_2] that is attenuated if N reserves in the soil are diminished over time leading to progressive N limitation (Norby et al. 2010; Reich et al. 2006). However, the mechanism of how the additional photosynthate is used to support growth and end of season biomass through its utilization in respiration is a source of controversy (Gonzalez-Meler et al. 2004). A more mechanistic understanding of plant respiratory responses to elevated [CO_2] is important for our ability to accurately predict future climate because respiration can re-release 40% of daily carbon gained by photosynthesis thereby heavily influencing carbon balance at the plant, ecosystem, and global scales (Atkin et al. 2010). Due to its importance across these scales many studies have been conducted and there has been much debate about the magnitude and direction of plant respiratory responses to elevated [CO_2] (Drake et al. 1997; Amthor 2000; Leakey 2009a, b). Unlike photosynthesis, respiration plays different key roles in cellular metabolism based on spatio-temporal variation in the needs of the different tissue types within a single plant (i.e. heterotrophic /autotrophic, day/night, etc.). Many of the assumptions and literature justifications about the magnitude and direction of change in of leaf respiratory responses to elevated [CO_2] revolve around changes in leaf N content. This is because leaf N content is assumed to be an excellent indicator of tissue respiration rates, a notion that is supported by broad metabolic scaling (e.g. Reich et al. 2007) and energy accounting exercises made about growth and maintenance costs of plant tissues (e.g. Penning De Vries 1975). The lack of a mechanistic understanding of the response to elevated [CO_2] has lead to respiration being treated rather simplistically, as either fixed fractions of daily carbon gain or scaled to C:N ratios in many of the most integrative dynamic global vegetation models for IPCC projections of biotic feedbacks on climate (Prentice et al. 2007). However, respiration is not always a fixed fraction of daily photosynthesis (Dewar 1999, Lambers et al. 2008). Despite the attention to the interaction between N availability and elevated [CO_2] on photosynthesis, few studies specifically concerned

with respiration have manipulated N availability directly, leading to conflicting results (Ryan 1991; Wullschleger et al. 1992; Thomas et al. 1993; Ziska and Bunce 1994; Curtis et al. 1995; Volin and Reich 1996; Will and Ceulmans 1997; Tjoelker et al. 1999). ***In order to investigate these interactions further, chapter 3 leveraged the molecular tools available for Arabidopsis thaliana, combined with a custom designed gas exchange system to ask if the physiological and gene expression responses of mature leaf respiration to elevated CO_2 are N dependent.***

Prior to the post-genomics era, Pritchard et al. (1999) suggested a conceptual model for plant growth and development in elevated [CO_2] that put meristem function and gene expression as the central response hub leading to altered plant architecture and biochemistry. Subsequent studies in the post-genomics era support this model. Within hours of mature Arabidopsis leaves being exposed to elevated [CO_2], changes were observed in gene expression and epidermal patterning in younger leaves which were not exposed to the elevated [CO_2] treatment, suggesting systemic signaling from older tissues (Lake et al. 2001; Coupe et al. 2006; Levine et al. 2009). Although much research has focused on these epidermal changes induced by differing [CO_2] concentration, little is understood about the effects of elevated [CO_2] on the respiratory machinery over the course of leaf development (Robertson et al. 1998a, b; Ainsworth et al. 2006). Previous studies conducted under elevated [CO_2] have separately revealed greater mitochondrial numbers per cell (Griffin et al. 2001) and an increased transcription of genes coding for respiratory proteins (Ainsworth et al. 2006; Leakey et al. 2009, Chapter 3), but the developmental timing and coordination of respiratory gene expression across leaf development has not been examined specifically. Understanding the interaction between leaf development and elevated [CO_2] may be especially important for respiration because a body of previous work on the response of leaf respiration to different temperatures suggests that full acclimation to varied temperatures requires the leaf to develop at that temperature (Atkin et al. 2001; Atkin and Tjoelker 2003; Armstrong et al. 2006). ***Given this body of evidence, in Chapter 4 I conducted time-course experiment that followed an individual Arabidopsis leaf cohort from primordia to maturity to ask how transcriptional reprogramming of respiration and a stimulation in respiration interacted with the leaf developmental program when plants are grown in elevated CO_2.***

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CHAPTER II: IMPAIRMENT OF C4 PHOTOSYNTHESIS BY DROUGHT IS EXACERBATED BY LIMITING NITROGEN AND AMELIORATED BY ELEVATED [CO2] IN MAIZE [1]

Abstract

Predictions of future ecosystem function and food supply from staple C_4 crops, such as maize, depend on elucidation of the mechanisms by which environmental change and growing conditions interact to determine future plant performance. To test the interactive effects of elevated [CO_2], drought and nitrogen (N) supply on net photosynthetic CO_2 uptake (*A*) in the world's most important C_4 crop, maize (*Zea mays*) was grown at ambient [CO_2] (~385ppm) and elevated [CO_2] (550ppm) with either high N supply (168 kg N ha^{-1} fertilizer) or limiting N (no added fertilizer) at a site in the U.S. Corn Belt. A mid-season drought was not sufficiently severe to reduce yields, but caused significant physiological stress, with reductions in: stomatal conductance (up to 57 %), *A* (up to 44 %) and the in-vivo capacity of phosphoenolpyruvate carboxylase (up to 58 %). There was no stimulation of *A* by elevated [CO_2] when water availability was high, irrespective of N availability. Elevated [CO_2] delayed and relieved both stomatal and non-stomatal limitations to *A* during the drought. Limiting N supply exacerbated stomatal and non-stomatal limitation to *A* during drought. However, the effects of limiting N and elevated [CO_2] were additive, so amelioration of stress by elevated [CO_2] did not differ in magnitude between high N and limiting N supply. These findings provide new understanding of the limitations to C_4 photosynthesis that will occur under future field conditions of the primary region of maize production in the world.

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[1] This chapter appeared in its entirety in the Markelz RJC, Strellner RS, Leakey ADB (2011) Impairment of C_4 photosynthesis by drought is exacerbated by limiting nitrogen and ameliorated by elevated [CO_2] in maize. Journal of Experimental Botany 62: 3235-3246. This article is republished with permission of the publisher.

Introduction

The C_4 plant functional type contributes ~25-30% of global terrestrial productivity, includes many of the world's worst weeds, and contributes ~40% of the world's grain harvest (Patterson 1995; Gillon and Yakir 2001; USDA-FAS 2005). The most globally important C_4 grain crop is maize (*Zea mays*), which is grown in over 160 countries and contributed ~712 of the 800 million metric tons of total C_4 grain harvest in 2006 (http://faostat.fao.org/). Maize production has been dramatically increasing since 1960 and it is predicted to outpace wheat and rice as the number one cereal crop by the year 2020 (Pingali 2001). In addition to C_4 species being ecologically and nutritionally important, many of the current and candidate biofuel crops possess C_4 photosynthesis, including sugarcane (*Saccharum officinarum*), maize (*Zea mays*), switchgrass (*Panicum virgatum*) and Miscanthus (*Miscanthus* x *giganteus*; Somerville et al. 2010). Therefore, it is increasingly important to understand C_4 responses to global environmental change in order to predict future ecosystem function, food availability, and energy security. However, current predictions are limited by inadequate understanding of how interactions with other environmental variables enhance or exacerbate C_4 photosynthetic responses to rising atmospheric CO_2 concentration ($[CO_2]$) (Ghannoum et al. 2000; Sage and Kubien 2003; Leakey 2009).

Theoretically, net CO_2 assimilation rates (A) of C_4 species should not be directly stimulated by elevated $[CO_2]$ under optimal growth conditions of temperature, water availability and nutrient supply (Ghannoum et al. 2000). This is because at current atmospheric $[CO_2]$ the CO_2 concentrating mechanism of C_4 plants results in saturating $[CO_2]$ for the Rubisco enzyme in the bundle sheath cells (von Caemmerer and Furbank 2003). This theoretical expectation is supported by experimental data from free-air CO_2 enrichment (FACE) studies of maize in the Midwest U.S., irrigated sorghum in the South West U.S., and *Paspalum dilatatum* in a New Zealand pasture (von Caemmerer et al. 2001; Wall et al. 2001; Leakey et al. 2006). This lack of a consistent, direct enhancement of photosynthesis and yield in C_4 species across a broad range of growing conditions diminishes the extent that elevated $[CO_2]$ will offset global yield loss resulting from other aspects of environmental change, even if elevated $[CO_2]$ acts locally to ameliorate stress associated with greater drought and temperature (Leakey 2009).

C_4 plants in natural and agricultural ecosystems frequently grow in conditions of limiting water availability and/or limiting N supply. Globally, water availability is a key factor limiting

plant productivity and crop yield (Boyer 1982; Churkina and Running 1998; Nemani et al. 2003; Gerten et al. 2004; Mu et al. 2007). Summer precipitation events in mid-continental areas are projected to decrease in volume and/or frequency (Giorgi et al. 2001; Kling et al., 2003; Weltzin et al. 2003), and higher temperatures across the world will increase crop water use and deplete soil moisture thereby resulting in a greater risk of droughts this century (Meehl et al. 2007). Although fertilizer use is rising to address the N limitation of many crops, there is economic and ecological pressure to limit fertilizer use in all regions (Wallace and Knausenberger 1997; Smil 1999; Galloway et al. 2008). Water and N limitation will be particularly acute in many developing countries of Africa and the Americas, which are characterized by: (1) heavy reliance on C_4 crops for food (Leakey, 2009), (2) the strongest links between local agricultural productivity and human well-being (Millennium Ecosystem Assessment, 2005), and (3) the most severe predicted impacts of global environmental change (Lobell et al. 2008).

The mechanisms determining photosynthetic performance can be evaluated in terms of non-stomatal and stomatal limitations through analysis of the response of A to intercellular [CO_2] (c_i), or A/c_i curves (Farquhar and Sharkey 1982; Lawlor and Cornic 2002; Long and Bernacchi 2003; Ghannoum 2009). Non-stomatal limitations to A include numerous biochemical and structural properties of leaves that are commonly quantified and modeled in terms of their effects on the capacities for: (1) carboxylation by PEPC (V_{pmax}), which determines the initial slope of the C_4 A/c_i curve, and (2) carboxylation by Rubisco as well as regeneration of PEP by PPDK, which each can limit the asymptote of the C_4 A/c_i curve (V_{max}; von Caemmerer 2000). Stomatal limitation to A determines the c_i at which A is operating on the A/c_i curve. It is quantified from A/c_i curves by comparing observed A with the value that would be achieved if there was no resistance to diffusion of CO_2 through the stomata from the atmosphere to intercellular leaf space (Farquhar and Sharkey 1982).

Elevated [CO_2] has the potential to play an important role in future C_4 plant performance if it relieves limitations to A that result from inadequate supplies of water and N. Elevated [CO_2] consistently ameliorates reductions in A caused by drought stress in C_4 species (Samarakoon and Gifford 1996; Ghannoum et al. 2000; Wall et al. 2001; Leakey 2009), but how stomatal and non-stomatal factors contribute to the response is still uncertain. For example, in some cases the initial slope of the A/c_i curve is lower in plants grown at elevated [CO_2] (e.g. Maroco et al. 1999; Watling et al. 2000; Driscoll et al. 2006), which would counteract amelioration of drought stress

resulting from reduced stomatal conductance and water use. Yet, in other situations the shape of the A/c_i curve does not change (e.g. von Caemmerer et al. 2001; Leakey et al. 2006). Historical improvements in yields of maize in the U.S. Corn Belt have been attributed in part to greater root growth supporting greater water capture (Hammer et al. 2009). Despite generally greater root:shoot ratios, limiting N supply can reduce root growth (Hocking and Meyer 1991) and thereby has the potential to prevent maize roots from accessing water deep in the soil during periods of low rainfall. Limiting N can increase leakiness of the C_4 cycle, and also reduce the capacity of key enzymes involved in the C_4 carbon concentrating mechanism and CO_2 fixation (Ranjith et al., 1995; Ghannoum and Conroy, 1998; von Caemmerer, 2000; Ghannoum et al., 2005), which could alter whether A remains CO_2-saturated at ambient [CO_2]. The effect of limiting N supply on C_4 photosynthetic and productivity responses to elevated [CO_2] have been studied under well-watered conditions (Hocking and Meyer 1991; Ghannoum and Conroy 1998), but the results were inconsistent.

The FACE facility at the University of Illinois at Urbana-Champaign in the Midwest U.S. allowed treatments of ambient [CO_2] and high N (ACHN), ambient [CO_2] and limiting N (ACLN), elevated [CO_2] and high N (ECHN), and elevated [CO_2] and limiting N (ECLN) to be imposed on maize growing under rain fed, open-air field conditions with an undisturbed soil-plant-atmosphere continuum; thereby avoiding the unintended artifacts on plant microclimate caused by experimental enclosure (Long et al. 2006; Ainsworth et al. 2008). In conjunction with a significant drought event in August 2008, this provided a rare opportunity to test the response of the model C_4 plant, maize, to the interactive effects of elevated [CO_2], drought and limiting N supply under field conditions. The low environmental and genetic variability of the study system also maximized the power of the experimental design to detect subtle treatment effects while testing the following hypotheses: (1) limiting N supply will reduce the capacities of the CO_2 concentrating mechanism and CO_2 fixation, causing a higher [CO_2] saturation point for A, and thus greater sensitivity of A to elevated growth [CO_2]; (2) growth at elevated [CO_2] will relieve both stomatal and non-stomatal limitations to A during periods of drought; (3) limiting N supply will exacerbate stomatal and non-stomatal limitation to A during drought, thereby enhancing the beneficial effects of elevated [CO_2].

Results

Rainfall and Soil Moisture

January to July total rainfall was the second greatest in 119 years and August rainfall was the sixth lowest on record (Illinois State Water Survey, http://www.isws.illinois.edu/atmos/statecli/cuweather/2008/aug2008.pdf). As a consequence, the seasonal course of soil volumetric moisture content ($H_2O\%_{v/v}$) was dominated by wet early-season conditions, a single extended drying event in the mid-season, and late-season rewetting of the soil (Fig. 1 A-C). On day of year (DOY) 190, soil $H_2O\%_{v/v}$ at all depths was near field capacity. Significant soil drying occurred from DOY 190-240, first in shallow depths, and then also in progressively deeper soil layers. This period of soil drying corresponded with the vegetative growth of the crop and the early stages of reproductive development (Figure 2.5). Significant rainfall between DOY 242 and 260 then returned soil $H_2O\%_{v/v}$ to field capacity during the later stages of reproductive development (Figure 2.1 A-C, Figure 2.5).

At the beginning of the season there was no difference in soil $H_2O\%_{v/v}$ between any of the treatments (Figure 2.1 A-C). However, both CO_2 and N treatments affected the rate at which soil moisture was depleted by crop water use. Consequently, for a significant fraction of the growing season both elevated [CO_2] and LN treatments resulted in greater soil $H_2O\%_{v/v}$, when considering the soil profile as a whole (Figure 2.1 A-C). The nature of these treatment effects varied between soil layers. At depths of 5-25 cm, soil $H_2O\%_{v/v}$ was significantly greater in ECLN than the other three treatments (Figure 2.1 A). Despite this, at the peak of the drought (DOY 235-239) no further drying of the soil at depths of 5-25 cm was achieved in any treatment, suggesting that all of accessible soil moisture had been exhausted by the plants in every treatment. In other words, a significant fraction of the root system in every treatment experienced soil water potentials near or at the permanent wilting point. At depths of 25-55 cm, the CO_2 and N treatment effects were additive. Consequently, the rank order of soil $H_2O\%_{v/v}$ in the four treatments was: ECLN > ECHN = ACLN > ACHN (Figure 2.1 B). At the peak of the drought (DOY 239), significant soil moisture extraction was still occurring at depths of 25-55 cm in all four treatments. At depths of 55 – 105 cm, only plants grown at ambient [CO_2] extracted significant soil moisture (Figure 2.1 C).

In-situ Diurnal Courses of Leaf Gas Exchange

Early in the growing season when soil $H_2O\%_{v/v}$ was close to field capacity (DOY 193 and 197), there were no significant differences in the diurnal course of *A* between any of the treatments (Figure 2.2). However, significant effects of CO_2 and N treatments emerged over time, coincident with the progressive development of soil moisture deficits and physiological indicators of drought stress. By midway through the drought period (DOY 220) *A* had decreased in all treatments compared to earlier in the growing season. This response was ameliorated by elevated [CO_2] at both levels of N, leading to ~18% greater *A* in ECHN and ECLN when compared to ACHN and ACLN (Figure 2.2). By the time soil water deficits were greatest (DOY 232), *A* had been reduced by up to 44 % relative to the non-drought conditions at the beginning of the growing season. The decline in *A* associated with increasing soil moisture deficit over time was again significantly ameliorated by elevated [CO_2], but now also exacerbated by LN. Consequently, the rank order of *A* at midday on DOY 232 in the four treatments was: ECHN > ACHN > ECLN > ACLN (Figure 2.2). At this time, *A* was 25% greater in ECHN compared to ACHN, and *A* was 23% greater in ECLN compared to ACLN.

Early in the season when soil $H_2O\%_{v/v}$ was close to field capacity (DOY 193 and 197), midday stomatal conductance (g_s) was significantly lower (-33 % on average) under elevated [CO_2] at both levels of N supply (Figure 2.2). By midway through the period of low rainfall (DOY 220) the magnitude of the CO_2 effect on g_s was greatly diminished (-19 % on average) because g_s had decreased much more over time in ACHN and ACLN than in ECHN and ECLN. By the time soil water deficits were greatest (DOY 232), g_s had been reduced by up to 57 % relative to the non-drought conditions at the beginning of the growing season, with the drought induced reduction of g_s being significantly greater in both the ambient [CO_2] and LN treatments.

On all four measurement dates elevated [CO_2] grown plants maintained greater c_i values when measured in the field, regardless of N treatment (Figure 2.2). During the period of most severe drought (DOY 232), LN treatments also had significantly lower c_i than HN treatments, consistent with changes in g_s.

A/c_i response curves

A/c_i curves (Figure 2.3) were measured in order to assess the stomatal and non-stomatal factors limiting *A*. Both the maximum rate of PEP carboxylation (V_{pmax}) and the [CO_2]-saturated rate of *A* (V_{max}) declined in all treatments as drought progressed over time. The decline in V_{max}

from DOY 204 to 228 was 13 %, on average and there were no effects of CO_2 or N treatments on V_{max} on any date (Figure 2.3). From DOY 204 to 228, increasing drought stress resulted in V_{pmax} declining by up to 58 %, with considerable variation among treatments. The non-stomatal limitation to *A* resulting from these reductions in V_{pmax} was ameliorated by elevated [CO_2] and exacerbated by LN. As a result of significant, additive CO_2 and N effects on V_{pmax}, the rank order of V_{pmax} in the four treatments on DOY 228 was: ECHN > ACHN = ECLN > ACLN (Figure 2.3). The first precipitation events greater than 2mm in over 20 days occurred on DOY 235 and 236 (Figure 2.1 A). Following this there were no longer any treatment effects on V_{pmax} (DOY 240).

Output from statistical analysis of *in-situ* c_i and A/c_i curves for each treatment on dates representing non-drought (DOY 197 and 204) and drought conditions (DOY 228 and 232) were combined in order to estimate stomatal limitation to *A* (Figure 2.4). Under non-drought conditions, there was almost no stomatal limitation to *A* (0.02-0.03) because mean c_i was at or above the inflexion point of the A/c_i curve. The development of significant water deficits caused stomatal limitation to *A* to increase many-fold in all treatments except ECHN, reaching a maximum of 0.49 in ACLN. Greater stomatal limitation under drought was ameliorated by elevated [CO_2], but exacerbated by LN. As a result, the rank order of stomatal limitation to *A* in the four treatments under drought stress was: ACLN > ACHN > ECLN > ECHN (Figure 2.4).

Leaf Area Index, Development, Biomass, and Yield

There were no significant effects of elevated [CO_2] on leaf area index (LAI), biomass, yield, or development (Table 2.2; Figure 2.5). However peak LAI was significantly lower in LN treatments compared to HN treatments, regardless of growth [CO_2] (Table 2.2). This was associated with the development of fewer leaves per plant under LN (Figure 2.5). As a consequence, total biomass, stover biomass (i.e. the remaining plant biomass after the ear is removed), and kernel number at reproductive stage 6 were significantly reduced in the LN treatments, with again no significant effect of [CO_2] on growth (Table 2.2). Individual kernel size did not vary with either growth [CO_2] or N treatment (Table 2.2).

Discussion

The 2008 growing season in Central Illinois featured a very wet spring followed by the sixth driest August on record. Reductions in g_s (up to 57 %), *A* (up to 44 %) and V_{pmax} (up to 58 %) between DOY 193 and 232 coincided with a substantial decline in soil $H_2O\%_{v/v}$ and are

consistent with the crop suffering significant physiological drought stress. Consequently, comparison of crop performance early and late in the growing season provided a rare opportunity to assess the mechanistic basis for C_4 photosynthetic responses to interactions between drought stress, N supply and growth [CO_2] under fully open-air field conditions. Consistent with previous experiments on maize at this site, there was no effect of elevated [CO_2] on *A* under conditions of high N supply in the absence of drought (Leakey et al. 2004; 2006). Contrary to our first hypothesis, limiting N supply did not alter leaf photosynthetic capacity and the CO_2-saturation point of *A*. Therefore, there was no stimulation of *A* by elevated [CO_2] when N was limiting and water availability was high. In accordance with our second hypothesis, elevated [CO_2] delayed and relieved both stomatal and non-stomatal limitations to *A* during periods of drought. With respect to our third hypothesis, limiting N supply exacerbated stomatal and non-stomatal limitation to *A* during drought. However, the effects of limiting N and elevated [CO_2] were additive, so the extent to which drought effects on *A* were ameliorated by elevated [CO_2] did not differ between high N and limiting N supply. These findings provide new mechanistic understanding necessary to improve model predictions of future C_4 photosynthesis, net primary productivity and crop yield across a diverse range of growing conditions. The CO_2 effects observed during reproductive developmental stages in 2008 can be attributed to interactions with episodes of drought rather than plant developmental events because: (1) no effect of elevated [CO_2] was observed at any developmental stage in the 2004 growing season that lacked any periods of drought stress (Leakey et al. 2006); and (2) in the 2002 growing season, during vegetative developmental stages, the ameliorating effects of elevated [CO_2] again coincided with periods of drought (Leakey et al. 2004).

Limiting N did not make *A* sensitive to elevated [CO_2] under non-drought conditions

The interaction between N supply and elevated [CO_2] is key to the future performance of C_3 species (Stitt and Krapp 1999, Poorter and Perez-Soba 2001, Reich et al., 2006; Rogers et al. 2009), but has been largely unexplored in C_4 species. Previous experiments at SoyFACE in which unstressed maize showed no photosynthetic response to elevated [CO_2] assessed plants receiving significant fertilizer inputs (168 kg N ha^{-1}; Leakey et al. 2004; 2006). Along with favorable climatic and edaphic conditions, the high rate of fertilizer application in Central Illinois results in maize yields that are amongst the greatest in the world (USDA-FAS 2005). Using FACE technology to test the effect of elevated [CO_2] on maize grown without fertilizer inputs

resulted in an experiment with greater relevance to the limiting N supply under which C_4 crops are grown in many other regions of the world (Leakey 2009). The photosynthetic capacity of C_4 species declines as leaf N content decreases (Ranjith et al. 1995; Ghannoum and Conroy 1998; Ghannoum et al. 2005), with the potential outcome that under limiting N the CO_2-saturation point of *A* would increase. If *A* became CO_2-limited in this manner then elevated [CO_2] would stimulate carbon gain and productivity under a broader range of growing conditions, i.e., both in the presence and absence of drought stress. Diurnal courses of *in-situ* leaf photosynthetic gas exchange and A/c_i curves measured early in the growing season (DOY 185 - 204) when adequate water was available to the crop revealed that, contrary to expectation, maize grown under the limiting N treatment produced leaves that were unaltered in terms of photosynthetic capacity and sensitivity to c_i. Instead of altering leaf physiological capacity, limiting N supply resulted in the production of fewer leaves, reduced LAI, biomass accumulation and yield compared to high N treatments. This provides a mechanistic explanation for the lack of any CO_2 effect on biomass accumulation during the vegetative growth stages of maize grown in pots of sand and supplied with a range of N from 0.5 to 25 mol m^{-3} NO_3 (Hocking and Meyer 1991). Together these results support the conclusion that the yield of maize, and probably other C_4 crops, will not be stimulated by rising [CO_2] this century across a wide range of soil fertility as long as they are not drought stressed.

Elevated [CO_2] delays and diminishes the stomatal and non-stomatal limitations to *A* that develop during the progression into drought stress

Drought is defined as when demand for water by a plant is not matched by water supply to the plant. In this study, drought stress is considered to be any physiological impairment resulting from the plant sensing or experiencing water deficits. Over the period of soil drying where surface soil $H_2O\%_{v/v}$ declined from near field capacity (DOY 193) to near the permanent wilt point (DOY 232), there were significant reductions in g_s (up to 57 %), *A* (up to 44 %) and V_{pmax} (up to 58 %). Many studies have reported the capacity of elevated [CO_2] to relieve drought-induced inhibition of *A*, growth, crop yield and net primary productivity (Samarakoon and Gifford 1996; Owensby et al. 1999; Ghannoum et al. 2000; Wall et al. 2001; Ottman et al. 2001; Leakey et al. 2004; Leakey 2009). The importance of both stomatal and non-stomatal limitations to *A* in causing reduced C_4 plant productivity under drought is also widely recognized, and has recently been comprehensively reviewed (Ghannoum 2009). However, there

is little information on the degree to which these mechanisms are engaged by drought and relieved by elevated [CO_2] under field conditions. This study provides evidence that in the primary region of maize production: (a) lower g_s at elevated [CO_2] results in reduced water use, slower depletion of soil $H_2O\%_{v/v}$ during periods of low rainfall, and a delay in the reduction of g_s and photosynthetic capacity by drought; and also (b) once drought stress is experienced by the plant at elevated [CO_2], decreases in g_s and V_{pmax} do not limit A as much as at ambient [CO_2] because c_i is greater.

Early season measurements when adequate water was available to the crop (DOY 185-204) did not detect any photosynthetic response to elevated [CO_2] in plants receiving either high N or limiting N. In all treatments, *in-vivo* measures of photosynthetic capacity (V_{pmax}, V_{max}) were at the upper range of those reported in the literature (e.g. von Caemmerer 2000; Driscoll et al. 2006) meaning that non-stomatal limitations to A were minimized. Likewise, g_s was high leading to c_i that were above the inflexion point of the A/c_i curve, resulting in essentially no stomatal limitation to A. During the mid-season period of low rainfall, plant water use caused soil $H_2O\%_{v/v}$ to decrease substantially, finishing near or at the permanent wilt point in shallow soil layers. The rate of soil drying was slower in elevated [CO_2] treatments, particularly in the middle (25 – 55 cm) and bottom (55 – 105 cm) soil layers. This slower soil drying at elevated [CO_2] was associated with smaller decreases in g_s, c_i and V_{pmax} over time in elevated [CO_2] compared to ambient [CO_2] treatments. This provides evidence that elevated [CO_2] delayed drought-induced stomatal and non-stomatal limitations to A. Once drought stress was experienced by plants in elevated [CO_2] treatments, the operating c_i was maintained near or above the inflexion point of the A/c_i curve, thereby reducing the negative effects on A of drought-induced reductions in the initial slope of the A/c_i curve (V_{pmax}) and g_s. In contrast, in ambient [CO_2] treatments, drought-induced reductions in V_{pmax} and g_s resulted in c_i that was below the inflexion point of the A/c_i curve. This was the cause of the greater reductions in A under ambient [CO_2].

Comparison of results from 2008 with data from previous growing seasons at the same field site suggests that elevated [CO_2] resulted in greater A by ameliorating episodic drought stress rather than affecting maize physiology during specific developmental events. During the 2002 growing season, A was greater under elevated [CO_2] compared to ambient [CO_2] during drought conditions, but not when drought stress was absent (Leakey et al. 2004). However, an important distinction was that in 2002 the drought occurred during early-season, vegetative

developmental phases and not during mid-season reproductive development as it did in 2008. In addition, during the drought-free growing season of 2004, there was no effect of elevated [CO_2] on *A* of maize at any developmental stage (Leakey et al. 2006). Across all three seasons the reduction in g_s at elevated [CO_2] was greatest during non-drought periods and diminished during periods of drought, irrespective of the developmental stage at which that occurred (Leakey et al. 2004; 2006). This is consistent with greater soil drying and drought sensitivity at ambient [CO_2]. Therefore, the observed changes in photosynthesis and g_s that explain the episodic treatment effects on *A* in 2008 are highly likely to result from progression through soil wetting and drying cycles rather than any effects on plant development or senescence.

The mechanism relieving drought stress via greater c_i is likely to be most important in situations where drought stress is prolonged. Under prolonged drought the delay in drought stress associated with lower water use and greater soil $H_2O\%_{v/v}$ at elevated [CO_2] would disappear as soil water resources in all treatments became exhausted. However, the relief of stomatal and non-stomatal limitations to *A* by greater c_i would remain. In contrast, the delay of drought stress will contribute more to overall amelioration of drought stress in situations featuring frequent wetting and drying cycles. Of course, at some point drought stress will be so severe that elevated [CO_2] will not have the capacity to sustain plant performance. This threshold will likely be a key tipping point in crop responses to climate change, but remains to be determined. The slow progression into drought stress that was observed (>40 days) emphasizes the importance of soil moisture holding capacity and a plant's capacity for proliferation of deep roots in determining the outcome of the elevated [CO_2] x drought interaction. Soils that are shallow or have a low moisture holding capacity, as well as pot-based experimental systems, may respond very differently which is supported by modeling analysis (Weng and Luo, 2008).

Limiting N exacerbates drought inhibition of *A*, but acts additively with elevated [CO_2]

Soil $H_2O\%_{v/v}$ was greater in limiting N treatments compared to high N treatments. However, extraction of water from shallow soil layers (5 – 25 cm) appeared to cease in all treatments when soils were at their driest in late August. This was not because demand for water had ceased, as significant soil drying was still occurring in deeper soil layers. Rather, this suggests that that limiting N supply constrained root growth and the capacity of the plants to extract all of the available water from a given volume of soil, causing greater drought stress despite smaller canopy size. This interpretation is consistent with the greater reductions in g_s, c_i,

V_{pmax} and *A* observed in limiting N treatments compared to high N treatments over the time that significant soil water deficits were developing. This type of interaction between N supply and drought stress may be favored in deep, high moisture holding soils such as the Midwest U.S. where deeper root growth can provide access to otherwise unused water resources. However, in conditions of lower soil water storage the outcome of the interaction might be reversed and drought stress will be more prevalent in productive genotypes or higher fertility conditions where shallow water resources can be exhausted more rapidly without the possibility of finding additional water deeper in the soil. This is consistent with Ghannoum and Conroy (1998) who observed greater *A* and biomass accumulation of *Panicum coloratum* and *P. antidotale* in response to elevated [CO_2] when grown at high N, but not under low N. In that study, plants grown at high N and ambient [CO_2] had c_i/c_a (~0.25) that was lower than is typical for unstressed C_4 species (~0.40). The enhancement of *A* by elevated [CO_2] at high N may therefore have been driven by amelioration of unintended drought stress caused by the high demand for water of pot-grown plants that were more than five times larger under high N than low N.

While limiting N exacerbated impairment of physiological function observed over the period of increasing soil water deficits, the effects of N supply and growth [CO_2] were additive. In other words, the extent to which elevated [CO_2] ameliorated drought stress did not vary with N supply. In combination with the finding that limiting N did not make *A* sensitive to elevated [CO_2] under non-drought conditions, this suggests that the nature of photosynthetic responses to elevated [CO_2] in maize should be consistent across a broad range of N supply. This study also adds to the evidence that elevated [CO_2] effects on *A* in C_4 species are strongly dependent on plant water status. In 2008, the amelioration of drought stress by elevated [CO_2] resulted in up to 25% greater rates of *A*. By comparison, in 2002, *A* was up to 41% greater under elevated [CO_2] than under ambient [CO_2] during a period of early season drought stress (Leakey et al. 2004). The greater impact of the drought on plant water status under ambient [CO_2] in 2002 was apparent from observations of leaf curling (Leakey et al. 2004), which did not occur in 2008. This may simply reflect a stronger drought in 2002 (minimum Palmer Crop Moisture Index = -1.19) versus 2008 (minimum Palmer Crop Moisture Index = -0.34), but could also be related to the reduced capacity of the root system early in the season to access deeper soil water.

The high water holding capacity of the deep soils at the SoyFACE site and moderate temperatures in August 2008 meant that, although rainfall was substantially below average for

>40 days, maize yield was not significantly reduced relative to favorable growing seasons (Leakey et al., 2006). Consequently, while this experiment revealed the mechanisms by which elevated [CO_2] ameliorated the drought-induced inhibition of *A* by stomatal and non-stomatal factors, the stress relief was not sufficiently sustained to result in significantly greater biomass accumulation or yield. It is important that yield at a site in the world's primary region of maize production was not enhanced by elevated [CO_2] in a year with a drought episode of moderate duration and intensity. Combined with no benefit of elevated [CO_2] in years lacking drought stress (Leakey et al. 2006), this contrasts significantly with the assumption in current models of future food supply predicting maize photosynthesis and yield will be consistently enhanced by elevated [CO_2] (see details in Tubiello et al. 2007a, b). Nevertheless, considerable uncertainty about the impact of global environmental change impacts on ecosystem goods and services from agricultural and natural ecosystems dominated by C_4 species will remain until C_4 species responses to elevated [CO_2] are examined across a much broader range of hydrological conditions than has been done to date.

Conclusion

This study revealed that elevated [CO_2] primarily exerts its effects on C_4 photosynthesis of maize by modulating how drought causes stomatal and non-stomatal limitations to *A*. Elevated [CO_2] delayed drought-induced reductions in g_s and V_{pmax} that inhibit *A*, while also relieving inhibition of *A* via greater c_i once drought stress induced reductions in g_s and V_{pmax}. Limiting N exacerbated drought stress. But, the degree to which drought stress was ameliorated by elevated [CO_2] did not differ between conditions of high N and limiting N supply. While elevated [CO_2] ameliorated inhibition of leaf-level photosynthetic carbon gain by drought, the effect was insufficient to drive any CO_2 effect on grain yield of maize under either high N or limiting N supply. This means that even accounting for moderate variations in soil fertility and drought stress, elevated [CO_2] appears not to enhance the yield of maize in its primary growing region. Further studies are needed to determine whether the CO_2-response mechanisms characterized here can relieve stress sufficiently to sustain yields of C_4 crops in times or places of severe drought.

Materials and Methods

Experimental design, cultivation, FACE system and crop growing conditions

During the 2008 growing season the SoyFACE experimental facility (www.soyface.illinois.edu) in Champaign, IL was used to test the effects of growth [CO_2] and N supply on *Zea Mays* cv 34b43 (Pioneer Hi-Bred International). The crop was planted on May 29, emerged on June 5, and was harvested on October 1. The experiment was laid out as a fully factorial, split-plot design in four experimental blocks (n = 4 for all statistical tests) with CO_2 treatment as the between-plot factor and N treatment as the split-plot factor. Each block contained one plot at current ambient [CO_2] (~385 ppm) and one plot at elevated [CO_2] (550 ppm). Half of each plot received standard N fertilization (168 kg N ha^{-1}, HN) while the other half received no N fertilization (LN). Both sub-plots had an estimated soil N credit of 45 kg N ha^{-1} from the soybean crop of the previous year. Soil N was measured on DOY (day of year) 198 to ensure continued lower N availability in the limiting N treatments. Fumigation operated from planting until harvest to a target [CO_2] of 550 ppm, which was chosen to simulate growing conditions projected to occur in 2050 (Prentice et al. 2001). In all other regards, the agronomic techniques, site management and fumigation technology used were the same as in previous experiments (Leakey et al. 2004; 2006). Air Temperature (T_{air}), relative humidity (RH%), rainfall, and incident photosynthetic photon flux density (PPFD) were measured by an on-site weather station as previously described (Leakey et al., 2004). At four locations in each subplot volumetric soil moisture content (H_2O%) was measured in 10-cm increments between depths of 5 to 105 cm every 3-5 days across the season using a capacitance probe (Diviner 2000, Sentek Sensor Technologies).

In-situ Leaf Photosynthetic Gas Exchange

Diurnal courses of in-situ photosynthetic gas exchange were measured on the youngest most fully expanded leaves of two plants in each subplot on 4 dates that corresponded to four developmental stages (Table 1). On each date, measurements began once dew had evaporated from leaf surfaces and continued at 2h intervals until just before sunset. Four open gas-exchange systems (Li-6400 and Li-6400-40; Li-COR) were used simultaneously and rotated among treatments and blocks to avoid sampling bias as described by Leakey et al. (2006a). Immediately before each time point, T_{air}, and incident PPFD were determined above the canopy. These conditions and growth [CO_2] were reproduced in the leaf chamber of the gas exchange systems

for all measurements during the timepoint. Leaf assimilation rate (A), stomatal conductance (g_s) and c_i were calculated following von Caemmerer and Farquhar (1981).

A/c_i Curves

The youngest most fully expanded leaves of two plants per subplot (8 plants total per treatment, from 4 replicate ambient or elevated [CO_2] plots) were harvested pre-dawn, re-cut under water and the cut surface kept immersed until measurements were completed. This was repeated on four dates corresponding to four different developmental stages (Table 1). A/c_i curves of the excised leaves were assessed in the laboratory using the gas exchange apparatus described in the previous section and the protocol of Bernacchi et al. (2005), with the following modifications. Measurements were performed at 27 ºC, 1,750 µmol m^{-2} s^{1} PPFD, and [CO_2] of 25, 50, 75, 100, 150, 200, 300, 400, 575, 800, and 1000. The A to c_i relationship at a c_i < 50 ppm was used to solve for V_{pmax} following von Caemmerer (2000). The horizontal asymptote of a four-parameter nonrectangular hyperbola was used as an estimate for V_{max}. Stomatal limitation to A was estimated (Fig. 4), as described by Long and Bernacchi (2005), using mean values of *in-situ* c_i in combination with A/c_i curves drawn using V_{pmax} and V_{max} parameter values that corresponded to statistically significant treatment effects on dates representing non-drought (DOY 197 and 204) and drought stressed conditions (DOY 228 and 232).

Development, Leaf Area Index, Biomass, Yield

Plant ontological development was monitored every 3-5 days throughout the life cycle of the crop and developmental stages were determined based on classifications given in Ritchie et al., (1993). Leaf Area Index (LAI) was measured at the developmental stage corresponding to maximum vegetative canopy leaf area (Ritchie et al. 1993) using a plant canopy analyzer (LAI-2000, LiCOR, Lincoln, NE, USA). At the end of the growing season six plants were harvested from each plot to assess above-ground biomass accumulation. Material was aggregated into three fractions (ears, leaves, and stalks) that were oven dried at 70°C before weighing to determine dry mass. Dried grain was shelled from the ears and weighed to determine seed yield. From this sample, three hundred random maize kernels were weighed to determine individual grain size.

Statistical Analysis

All analyses were performed on plot means (N=4) in SAS (SAS 9.1, SAS Institute, Cary, NC) using the MIXED procedure with the Kenward-Rogers option. A threshold of $P < 0.1$ was used to determine statistical significance for this field study. In all cases, block was a random

effect, while [CO_2] and N treatments were fixed effects. The [CO_2] treatment was tested as the between-plot factor and N was tested as the within-plot factor. Averages of H_2O% in three layers of the soil profile (5 – 25 cm, 25 – 55 cm, 55 – 105 cm) were independently analyzed with DOY as a repeated measure and early season saturated soil $H_2O_{v/v\%}$ as a covariate. For all gas exchange parameters, data from different DOY were tested independently. For *in-situ* photosynthetic gas exchange, time of day was treated as a repeated measure of time.

Tables and Figures

Table 2.1 Calendar date, Julian day of year (DOY), and days after emergence (DAE) of diurnals and ACi curves with the corresponding developmental stages as defined by Ritchie et al., (1993) for field grown maize under either ambient (385 μmol mol^{-1}; AC) or elevated [CO_2] (550 μmol mol^{-1}; EC) and either high nitrogen supply (HN) or limiting nitrogen supply (LN) during 2008 at SoyFACE.

Date	DOY	DAE	Type	Developmental Stage			
				ACHN	ACLN	ECHN	ECLN
July 3	185	29	A/c_i	Leaf 7	Leaf 6	Leaf 7	Leaf 6
July 11	193	37	Diurnal	Leaf 10	Leaf 9	Leaf 10	Leaf 9
July 15	197	41	Diurnal	Leaf 12	Leaf 10	Leaf 12	Leaf 11
July 22	204	48	A/c_i	Leaf 16	Leaf 14	Leaf 17	Leaf 15
August 7	220	64	Diurnal	Blister Kernel	Blister Kernel	Blister Kernel	Blister Kernel
August 14	227	71	A/c_i	Milky Kernel	Milky Kernel	Milky Kernel	Milky Kernel
August 19	232	76	Diurnal	Milky Kernel	Milky Kernel	Milky Kernel	Milky Kernel
August 27	240	84	A/c_i	Dough Kernel	Dough Kernel	Dough Kernel	Dough Kernel

Table 2.2 Final total, stover, grain biomass, kernel number, individual kernel mass, and peak Leaf Area Index (LAI), for each of the maize plots grown under ambient [CO_2] High Nitrogen (ACHN), ambient [CO_2] Low Nitrogen (ACLN), elevated [CO_2] High Nitrogen (ECHN), and elevated [CO_2] Low Nitrogen (ECLN) at SoyFACE in Urbana, Illinois. Different letters indicate significant treatment differences (p-value <0.1).

Parameter	ACHN	ACLN	ECHN	ECLN
Total Biomass R6 (g plant $^{-1}$)	256±21^{a}	207±21^{b}	268±21^{a}	209 ±21^{b}
Stover Biomass R6 (g plant $^{-1}$)	118 ±8^{a}	97±8^{b}	114±8^{a}	96±8^{b}
Grain Biomass R6 (g plant $^{-1}$)	137±11^{a}	110±11^{b}	139±14^{a}	114±11^{b}
Kernel Number (plant $^{-1}$)	515±46^{a}	428±46^{b}	574±46^{a}	456±46^{b}
Individual Kernel Mass (mg)	266 ±10^{a}	257 ±10^{a}	267 ±10^{a}	250 ±10^{a}
Peak LAI	4.3 ±0.2^{a}	3.7 ±0.2^{b}	4.5 ±0.21^{a}	3.76 ±0.21^{b}

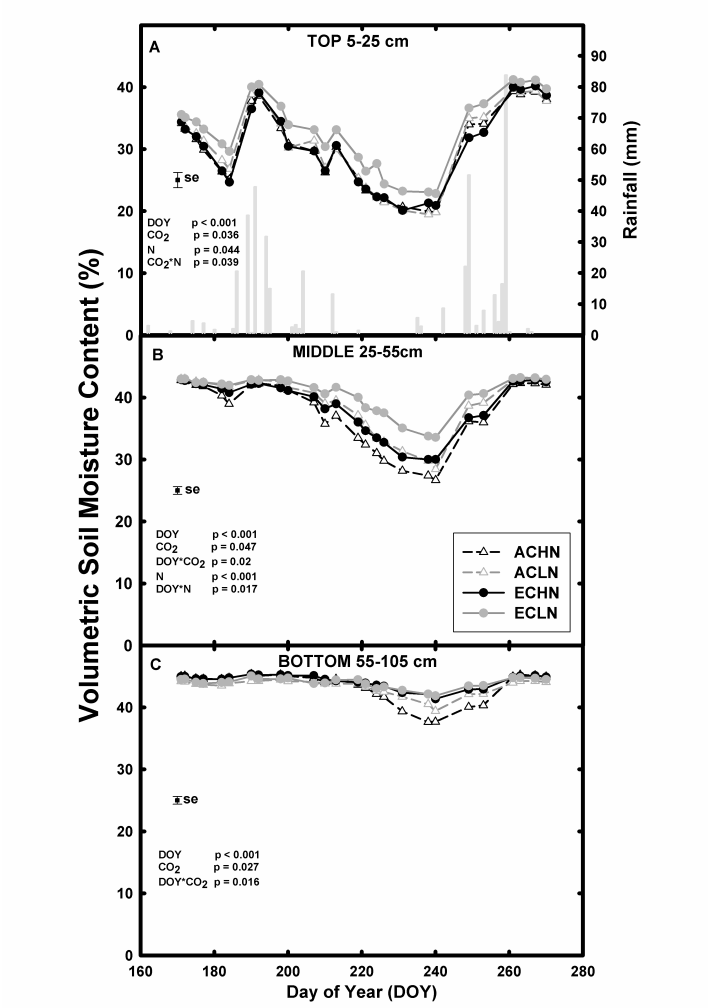

Figure 2.1 Volumetric soil moisture (%) measured from depths of 5-25 cm (A, TOP), 25-55cm (B, MIDDLE), 55-105 cm (C, BOTTOM) in plots of maize grown under ambient [CO_2] and high nitrogen (ACHN, open black triangles), ambient [CO_2] and limiting nitrogen (ACLN, open grey triangles), elevated [CO_2] and high nitrogen (ECHN, closed black circles), and elevated [CO_2] and limiting nitrogen (ECLN, closed grey circles) during the 2008 growing season at SoyFACE. Each point is the mean of the replicate plots (n=4) measured at that time, with the corresponding standard error calculated from the repeated measures ANOVA represented by the bars around the closed black box plotted on the lower left of each panel. Statistically significant treatment effects ($P<0.05$) are listed in each panel. Precipitation per day (mm) is shown as grey bars in panel A.

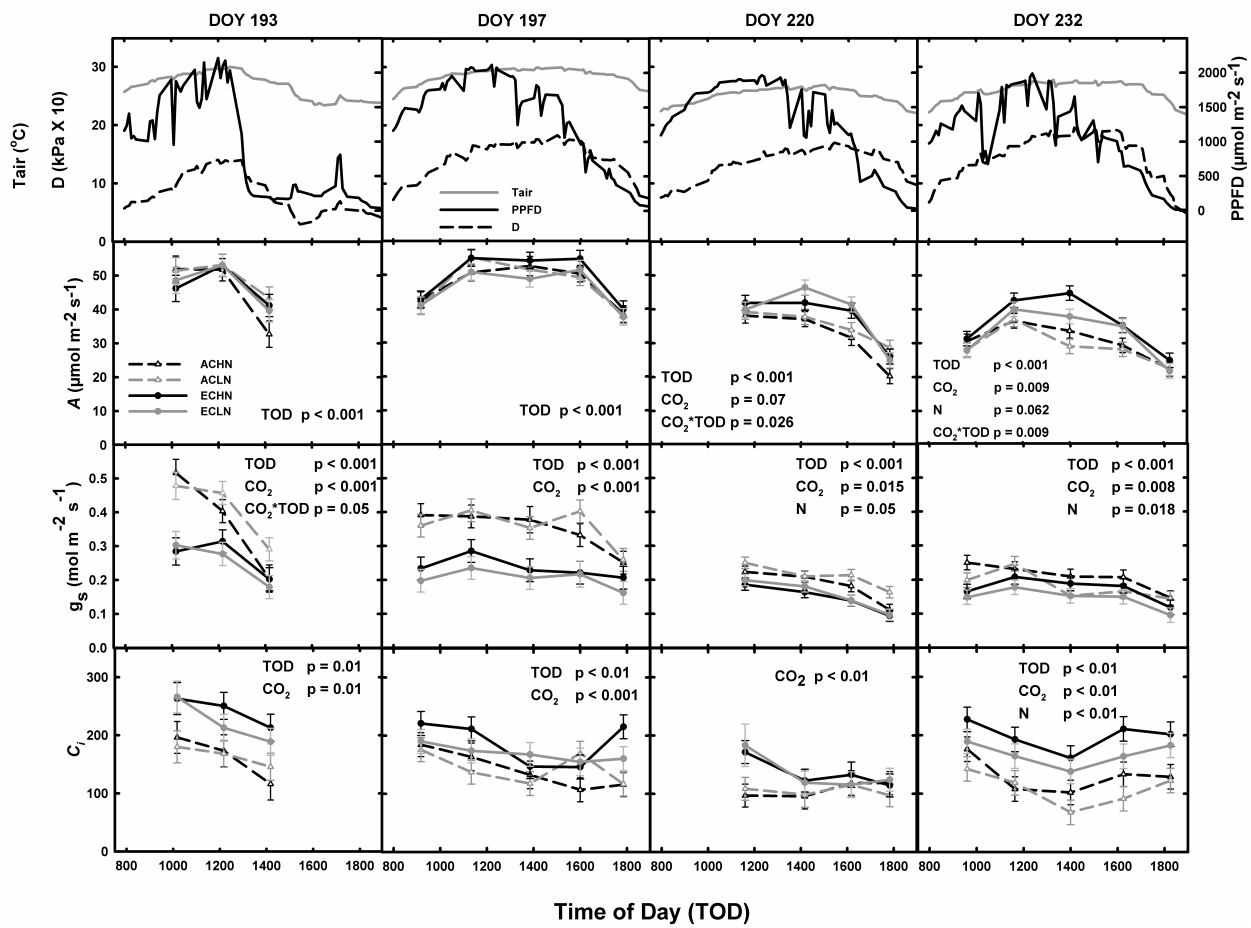

Figure 2.2 In-situ diurnal courses of Tair, vapor pressure deficit (D), PPFD, A, gs, and ci of the youngest fully expanded leaf of maize grown under ambient [CO_2] and high nitrogen (ACHN, open black triangles), ambient [CO_2] and limiting nitrogen (ACLN, open grey triangles), elevated [CO_2] and high nitrogen (ECHN, open grey triangles), and elevated [CO_2] and limiting nitrogen (ECLN, closed grey circles) on four dates (DOY) during the 2008 growing season at SoyFACE. Each point is the mean (± se) of the replicate plots measured at that time point (n=4). Statistically significant treatment effects ($P<0.05$) are listed in each panel.

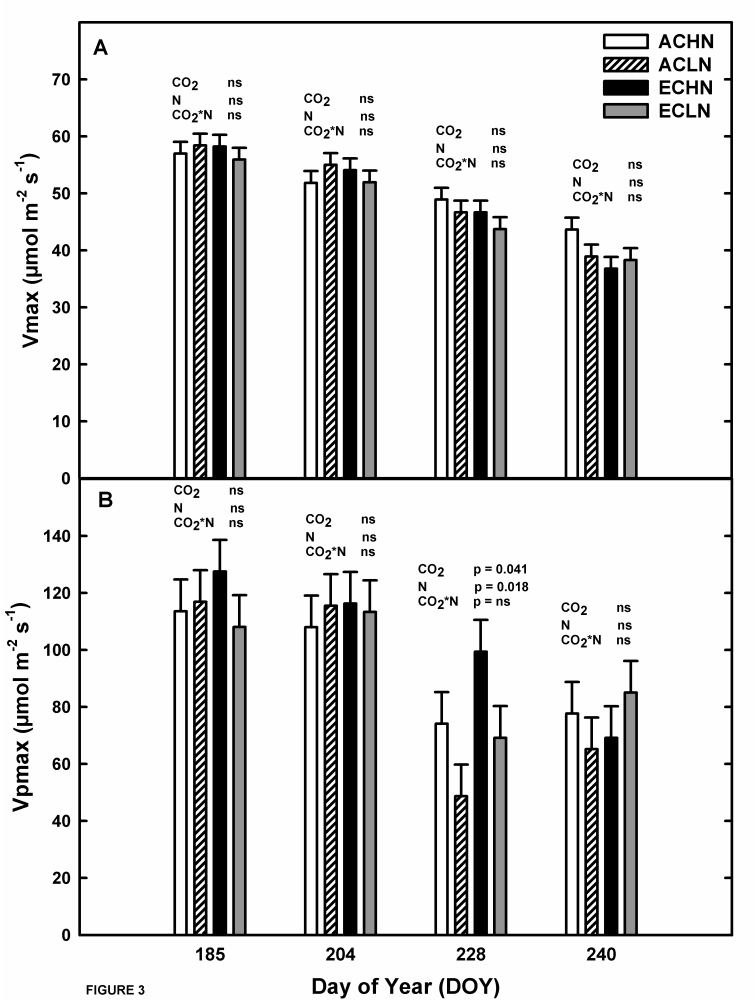

Figure 2.3 (A) Asymptote of A/ci curve (Vmax) and (B) maximum carboxylation capacity of PEPC (Vpmax) of the youngest fully expanded leaf of maize grown under ambient [CO_2] and high nitrogen (ACHN, white bars), ambient [CO_2] and limiting nitrogen (ACLN, hatched bars), elevated [CO_2] and high nitrogen (ECHN, black bars), and elevated [CO_2] and limiting nitrogen (ECLN, grey bars) on four dates during the 2008 growing season at SoyFACE. Each point is the mean (± se) of the replicate plots measured at that time point (n=4). The statistical significance of CO_2, N and CO_2 x N effects within each DOY are indicated (ns = not significant).

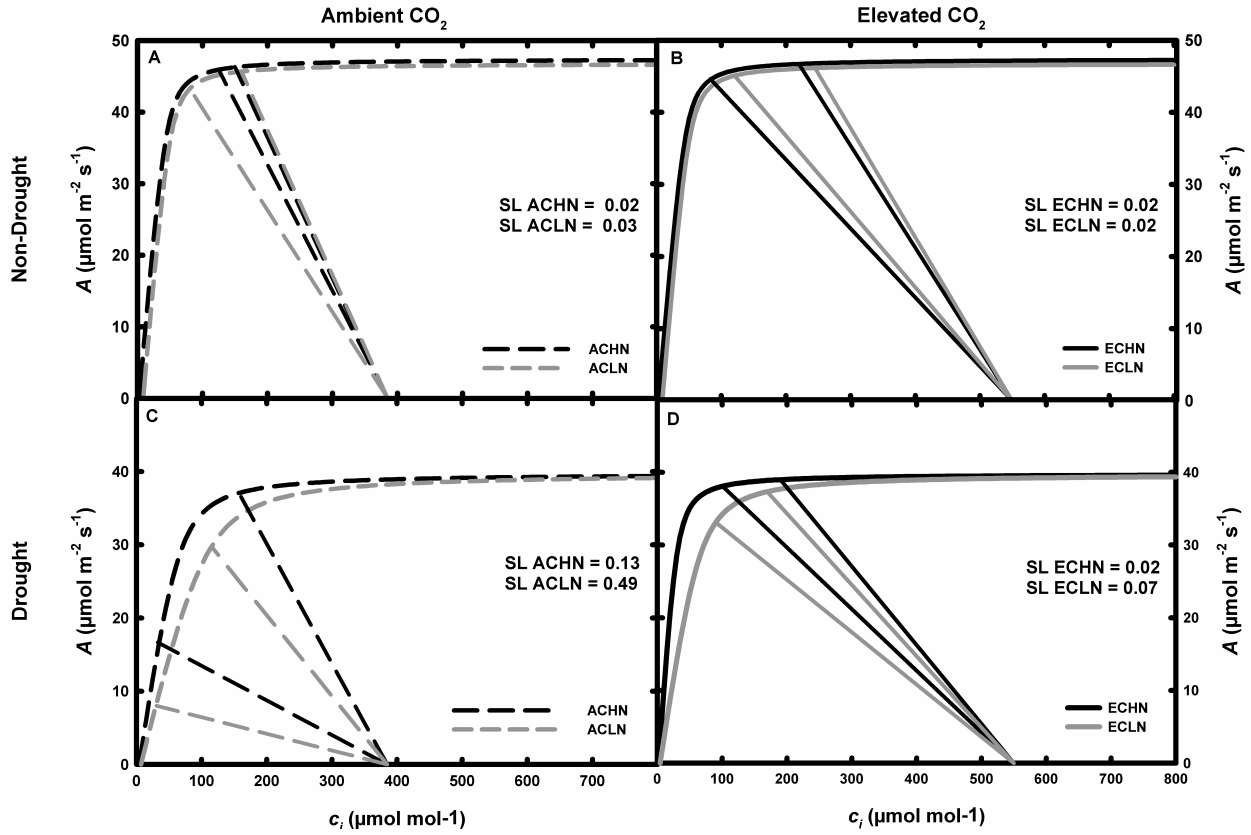

Figure 2.4 Summary of A/c_i response curves and CO_2 supply functions for maize grown at ambient [CO_2] (Panels A and C, dashed lines) and elevated [CO_2] (Panels B and D, solid lines) as well as high N (black lines) and limiting N (grey lines) during non-drought conditions (panels A and B) or drought conditions (panels C and D). A/c_i response curves represent statistically significant treatment effects for values of Vpmax and Vmax (n = 4; see Figure 2.3) under non-drought conditions (DOY 204) and drought conditions (DOY 228). Superimposed are supply functions representing the maximum and minimum of ci observed at midday in the field (see Figure 2.2) under non-drought conditions (DOY 197) and drought conditions (DOY 232). Estimates of stomatal limitation (SL) using mean midday ci in each treatment are reported in each panel.

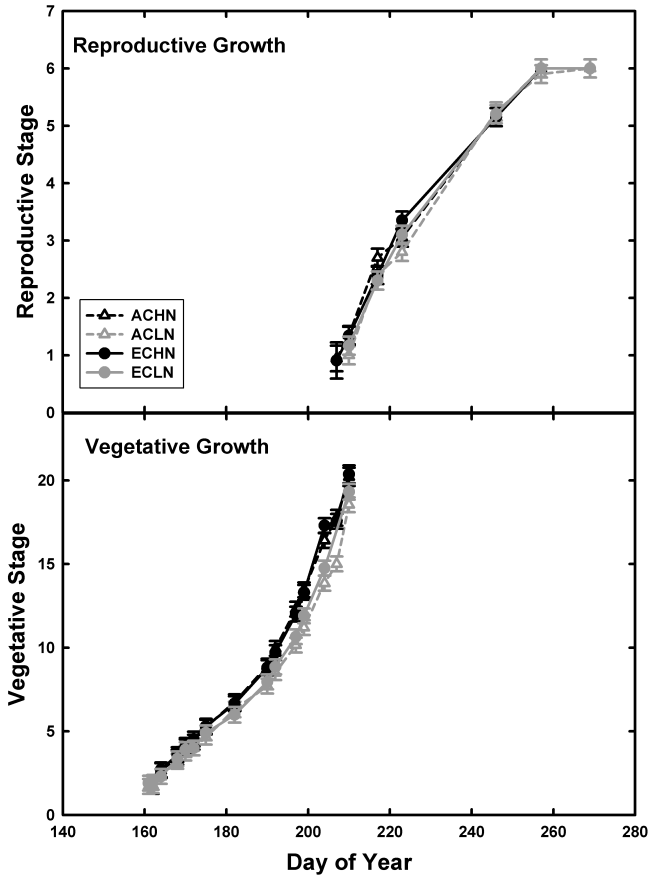

Figure 2.5 A graph showing the progression of vegetative and reproductive development for maize grown at either ambient or elevated [CO_2] and either high or limiting N availability during the 2008 growing season at SoyFACE.

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CHAPTER III: TRANSCRIPTIONAL REPROGRAMMING AND STIMULATION OF LEAF RESPIRATION BY ELEVATED [CO2] IS DIMINISHED, BUT NOT ELIMINATED, UNDER LIMITING NITROGEN SUPPLY

Abstract

The effects of elevated [CO_2] on plant respiration have been studied for the past twenty-five years without a consensus about the magnitude, direction, or mechanism of response. Positive effects of elevated [CO_2] on respiration of mature leaves have been attributed to greater substrate supply resulting from stimulated photosynthetic CO_2 assimilation. Negative effects of elevated [CO_2] on leaf respiration have been attributed to reduced demand for energy from protein turnover assumed to result from lower leaf N content. *Arabidopsis thaliana* was grown in ambient (370 ppm) and elevated (750 ppm) [CO_2] with limiting and ample N availabilities to test the hypothesis that varying N supply alters the relative strength of these two opposing, but not mutually exclusive, mechanisms. The stimulation of leaf dark respiration was attenuated under limiting N (+12%) compared to the ample N supply (+30%). This response was associated with smaller stimulation of photosynthetic CO_2 uptake but not interactive effects of elevated CO_2 and N supply on leaf protein, amino acid content or specific leaf area. Elevated [CO_2] also resulted in greater abundance of transcripts encoding many components of the respiratory pathway. A conserved mechanism of transcriptional reprogramming to regulate sink-source balance at elevated [CO_2] has now been observed across a wide range of herbaceous species. The greatest transcriptional response to elevated [CO_2] was observed under ample N supply at midday versus midnight, consistent with previous reports that protein synthesis in Arabidopsis are greatest during the day and transcriptional control of carbon metabolism interacts with the circadian clock.

Introduction

Atmospheric CO_2 concentration ($[CO_2]$) is increasing due to anthropogenic emissions of ~10 Pg of carbon each year (Canadell et al. 2007). The amount of anthropogenic CO_2 released into the atmosphere is relatively small compared to the 50- 60 Pg carbon that is released each year through terrestrial plant respiration (Prentice et al. 2001). Plant respiration can re-release 30-80% of carbon fixed through photosynthesis while providing the C skeletons and energy needed to support plant growth and maintenance (Atkin and Tjoelker 2003). Plant requirements for C skeletons and energy can vary spatially across tissues (Reich et al. 1998), daily between light and dark cycles (Hurry et al. 2005), developmentally (Armstrong et al. 2006) and in response to changing environmental conditions (Amthor 2000). Due to its importance at the plant, ecosystem and global scales, there has been much debate about the magnitude and direction of plant respiratory responses to elevated $[CO_2]$ (Drake et al. 1997; Amthor 2000; Leakey 2009a, b), and key synthesis papers have variously concluded that leaf respiration at elevated $[CO_2]$ increases, decreases, or does not change (Drake et al. 1999, Wang and Curtis 2002, Gifford 2003, Davey et al. 2004, Gonzalez-Meler et al. 2004). In this body of literature there are primarily two mechanisms discussed by which nighttime leaf respiration could change under elevated growth $[CO_2]$: (1) photosynthesis is stimulated in elevated growth $[CO_2]$, which leads to greater carbohydrate concentrations that could stimulate dark respiration due to greater supply of respiratory substrate; (2) growth in elevated $[CO_2]$ can reduce leaf nitrogen concentration, which is often accepted as a proxy for reduced demand on dark respiration to support protein turnover at night (Amthor 1991; Ryan 1991; Gonzalez-Meler et al. 2004). These opposing, but not mutually exclusive, influences on dark respiration make it very difficult to predict leaf dark respiratory responses to climate change factors making dark respiration one of the largest knowledge gaps in climate change modeling (Atkin et al. 2010).

Cross talk between C and N metabolism at the biochemical and transcriptional level is essential for supporting maximal growth on limited N resources (Hirel et al. 2007; Lea and Azevedo 2007; Tschoep et al. 2009), and is a well-recognized driver of photosynthetic and biomass responses to elevated $[CO_2]$. Limiting N availability reduces the stimulation of photosynthesis by elevated $[CO_2]$ because excess photoassimilate availability triggers a sugar-signaling feedback that reduces expression of photosynthetic genes, especially for Rubisco, reallocating photosynthetic N reserves to other sinks where they are needed for biosynthesis

(Moore et al. 1999; Rolland et al. 2002; Stitt and Krapp 1999; Ainsworth and Long 2005; Ainsworth and Rogers 2006; Leakey et al. 2009b). Elevated [CO_2] decreases leaf N concentration, partly due to dilution by larger carbohydrate pools and partly as a result of changes in N acquisition and allocation – with the effect being greater as the N supply becomes increasingly limiting (Ainsworth and Long 2005, Taub and Wang 2008). Many studies have examined plants growing under varied elevated [CO_2] levels and N availabilities and have discovered much about the mechanistic basis of photosynthetic, biomass and yield responses to elevated [CO_2] (Conroy and Hocking 1993; Weber et al. 1994; Lloyd and Farquhar 1996; Rogers et al. 1996a, b; Farage et al. 1998; Geiger et al. 1999). However, the role of the N supply in determining respiratory responses to elevated CO_2 remains unclear (Gifford 2003; Gonzalez-Meler et al. 2004). A number of studies have examined the relationship between N and respiration by using correlative approaches to link leaf N to respiration rates across species (Ryan 1991; Wullschleger et al. 1992; Thomas et al. 1993; Ziska and Bunce 1994; Will and Ceulmans 1997; Tjoelker et al. 1999). However, very few studies have quantified dark respiration responses to elevated CO_2 under varying levels of N supply, but of those studies that have examined the interaction have focused on trees, producing conflicting results (Curtis et al. 1995; Volin and Reich 1996).

Substrate supply is proposed to control respiratory capacity in the long-term while demand for energy and carbon skeletons determines respiration rates in the short-term (Williams and Farrar 1992). Recent molecular and physiological evidence from plants grown at elevated [CO_2] in the field lends support to the Williams and Farrar hypothesis by showing that greater photoassimilate supply was associated with transcriptional up-regulation of the respiratory pathway and greater respiratory flux in both soybean and rice grown under elevated [CO_2] (Leakey et al. 2009a; Fukayama et al. 2011). Greater photosynthetic carbon gain could also be associated with greater demand for energy necessary to support phloem loading as additional photoassimilates are exported to sink tissues to support greater growth (Korner et al. 1995; Komor 2000). This would represent a significant modification to the leaf energy budget as phloem loading is estimated to account for 29% of nighttime energy demand (Bouma et al. 1995). However, soybean is a legume and rice is grown with heavy N inputs. These two studies are examples of plant growth under ample N conditions where both the greatest stimulations in photosynthesis and small or no reductions in leaf N concentration in elevated [CO_2] are observed

(Stitt and Krapp 1999; Ainsworth and Long 2005). N metabolism and protein turnover are intrinsically linked to respiration because C skeletons are needed to incorporate inorganic N into organic amino acids (Fernie et al. 2004; Palencher et al. 2004; Plaxton and Podesta 2006) and respiration derived energy is needed for protein turnover (Bouma et al. 1994; Amthor 2000; Gifford 2003). Therefore it has been proposed that some of the reported variability in respiratory responses to elevated [CO_2] may then relate to plant N status – where plants growing with limiting N supply may have reduced protein turnover at elevated [CO_2] (Amthor 1989; Drake et al. 1999; Gonzalez-Meler et al. 2004). This would reduce the demand for respiratory products and attenuate or eliminate changes in respiratory flux, despite greater photoassimilate availability. Under such circumstances, transcriptional reprogramming for greater respiratory capacity would be of no adaptive benefit. Poplar has been grown without significant fertilization at elevated [CO_2] in two Free Air Concentration Enrichment (FACE) experiments. In both cases, there was no evidence of transcriptional reprogramming of respiration in developing or mature leaves prior to the onset of senescence (Gupta et al. 2005; Taylor et al. 2005; Cseke et al. 2009, Tallis et al. 2010). Neither was a significant effect of elevated [CO_2] on leaf respiration in the dark detected (Davey et al. 2004; Loreto et al. 2007). The contrasting responses of soybean and rice versus poplar suggest that a direct comparison of the genome-wide transcriptional response in leaves to elevated [CO_2] under ample and limiting N coupled to biochemical and physiological analysis could provide an valuable initial step towards understanding the complex signaling and metabolic responses regulating leaf respiration at elevated [CO_2]. The current study tested leaf dark respiratory responses to elevated CO_2 in Arabidopsis under ample versus limiting N availability. The use of Arabidopsis is advantageous for asking mechanistic questions regarding [CO_2] and N interactions due to the availability of: (i) genomic tools and existing knowledge of transcriptional and biochemical regulation of C and N metabolism (Scheible et al. 2004); (ii) clearly defined limiting N treatments (Tschoep et al. 2009); and (iii) detailed previous work regarding whole plant responses to elevated CO_2 (Teng et al. 2006; Li et al. 2008). A great majority of work on Arabidopsis focuses on entire rosette tissue, instead of individual leaves, and an individual leaf approach has been demonstrated to better resolve molecular responses to mild treatments that might have been otherwise masked by using whole rosettes (Skirycz et al. 2010). This approach was used to test the hypotheses that elevated CO_2 and N supply interact in mature leaves so that: (1) under ample N supply, greater photoassimilate availability and no change in

leaf N and protein content at elevated [CO_2] will be associated with transcriptional reprogramming of respiration to support greater respiratory flux; (2) under limiting N supply, a reduction in leaf N and protein content at elevated [CO_2] will counteract greater photoassimilate availability such that transcriptional reprogramming of respiration is not observed and any stimulation of respiration rate is attenuated or eliminated.

Results

Biomass, Photosynthesis, Respiration and Leaf Biochemistry

The stimulation of biomass by elevated [CO_2] was significantly smaller under limiting N (47%) compared to the ample N supply (63%; Figure 3.1 A). Likewise, the stimulation of light-saturated photosynthetic CO_2 assimilation (A_{sat}) by elevated [CO_2] was significantly smaller under limiting N (61%) compared to the ample N supply (82%; Figure 3.1 B), consistent with a large number of previous experiments. There was a detectable stimulation of nighttime leaf respiration by elevated [CO_2] under both ample and limiting N supplies, but the effect was smaller under limiting N (+12 %) than ample N (+30 %; Figure 3.1 C). In contrast to the interactive effects of CO_2 and N supply on A_{sat}, respiration and biomass, the responses of specific leaf area (SLA) as well as leaf carbohydrate, protein, and amino acid pools to elevated [CO_2] did not vary with the level of N supply (Figures 3.2 and 3.8). At midnight, elevated [CO_2] led to 50% greater leaf starch content and 24% greater sugar content on average across limiting N and ample N treatments (Figure 3.8 A-B). At the same time, there was no significant effect of elevated [CO_2] on leaf soluble protein or free amino acid contents per unit leaf area in limiting N or ample N treatments (Figure 3.2 C-D). Elevated [CO_2] led to a lower leaf protein and amino concentrations on a dry mass basis to a similar degree in the limiting and ample N treatments, respectively (Figure 3.8 B-C). The decrease in protein and amino acid concentrations at elevated [CO_2] were approximately in proportion to changes in SLA, which also did not differ in magnitude between ample N and limiting N treatments (Figure 3.8 A). The limiting N treatment caused a significant increase in the sucrose to amino acid ratio and within each level of N, while elevated CO_2 caused a significant increase in the ratio relative to the ambient treatment (Figure 3.2 E). Leaf protein and amino acid contents per unit leaf area were greater under ample N compared to limiting N supply (Figure 3.2 C-D), but this was not associated with any N supply effects on SLA (Figure 3.8 A). Distinct from leaf protein and the other leaf chemistry parameters assessed, only leaf N concentration showed a significant elevated CO_2 by N supply interaction

response (Figure 3.2f). Elevated [CO_2] led to 20% lower leaf N content in the limiting N treatment, but had no effect in the ample N treatment (Figure 3.2 F).

Transcript Profiles

The Arabidopsis chip used to analyze gene expression represented 24,000 genes. Of the 12,826 gene transcripts present in at least 3 replicate samples from every treatment, 4439 had significant differences in abundance between ambient [CO_2] and elevated [CO_2], 1708 transcripts differed significantly in abundance between limited N and ample N supplies, and 8640 transcripts differed significantly in abundance between midday and midnight (TOD) and 258 transcripts had a significant CO_2 by N interaction (Table 3.1). Hierarchal clustering of the intensity values (log_2) of the significantly responding genes for each of the treatments demonstrates that transcripts responding significantly to elevated CO_2 generally responded in the same direction regardless of N treatment (Figure 3.3). However, transcripts that significantly responded to elevated CO_2 during the day tended to respond more in the ample N treatment compared to the limiting N treatment, and this trend was not apparent at night (Figure 3.4). The stimulation of respiration in elevated [CO_2] was associated with greater abundance of transcripts encoding components of glycolysis, the TCA cycle and mitochondrial electron transport chain including three of the four CO_2 producing steps of the TCA cycle, and genes encoding mitochondrial protein import complexes during both the midday and midnight time points in both ample N and limiting N treatments (Figures 3.5 and 3.6).

Discussion

This experiment reproduced the interactive effects of elevated [CO_2] and N supply observed in many previous studies, where the stimulation of photosynthesis and biomass accumulation by elevated [CO_2] was attenuated by limiting N supply (Stitt and Krapp 1999; Ainsworth and Long 2005; Reich et al. 2006). In addition, leaf N concentration was significantly reduced by elevated [CO_2] in plants grown with a limiting N supply, but not in plants grown with ample N supply. This provided the appropriate context for investigating how N supply impacts respiratory responses to elevated [CO_2]. Elevated [CO_2] stimulated leaf respiration at night, and the response was attenuated with limiting N supply (+12 %) compared to ample N supply (+ 30%). This provides new evidence that variation in plant N status is likely to have contributed to the substantial variability in respiratory responses to elevated [CO_2] previously described in the literature (Drake et al. 1999; Wang and Curtis 2002; Gifford 2003; Davey et al. 2004; Gonzalez-

Meler et al. 2004). In addition, with limiting N supply, the response of leaf respiration at night to elevated [CO_2] was modest in relative terms (+12 %) and very small in absolute terms (0.1 μmol m^{-2} s^{-1}). This was detectable through the use of a custom-built gas exchange system. This CO_2 effect likely would not have been statistically resolved using the commercially available gas exchange systems used in most prior studies, which has probably compounded the challenge of understanding biological variation in respiration responses to elevated [CO_2]. Nevertheless, once integrated over the leaf canopy and time, small changes in respiration have the potential to significantly impact leaf, plant and ecosystem carbon balance (Poorter et al. 1990; Drake et al. 1999; Atkin and Tjoelker 2003; Gifford 2003). The abundance of transcripts encoding many components of the respiratory pathway was greater at elevated [CO_2] under both ample N and limiting N supplies in Arabidopsis. This extends the evidence of transcriptional reprogramming of respiration to elevated [CO_2] to include a non-leguminous dicot species in addition to a legume (Leakey et al. 2009; soybean) and a non-leguminous monocot (Fukayama et al. 2011; rice). Together these findings suggest the existence of a conserved transcriptional mechanism across a wide range of herbaceous species that helps to maintain sink-source balance within leaves in the manner proposed by Farrar and Williams (1990). This study takes an initial step towards resolving the details of this mechanism, and addressing the major uncertainty surrounding the role of respiration in driving plant and ecosystem responses to global environmental change (Amthor 2000; Atkin et al. 2010).

In accordance with our first hypothesis, under ample N supply, growth at elevated [CO_2] led to greater photoassimilate availability, no change in N concentration, no change in leaf protein content per unit leaf area, greater abundance of transcripts encoding components of the respiratory machinery, and greater rates of leaf respiration at night. Our second hypothesis was not fully supported by the data. There was an interaction effect of elevated [CO_2] and N supply on respiration, in which the stimulation of respiration at elevated [CO_2] was smaller (+12 %) with limiting N supply than with ample N supply (+30 %). However, this attenuated response did not appear to result from reduced plant nitrogen status counteracting the influence of greater photoassimilate availability, as was predicted. These findings are evaluated below with consideration of treatment effects on: (a) substrate supply for respiration; (b) demand for C skeletons and energy from respiration; (c) leaf N as a proxy for leaf protein status; and (d) transcriptional regulation of respiratory capacity.

It is widely accepted that the stimulation of photosynthetic CO_2 uptake by elevated [CO_2] will generate a greater supply of carbohydrate substrate for respiration and that this response is observed across a wide range of species and environmental conditions (Drake et al. 1997; Drake et al. 1999; Gifford 2003; Gonzalez-Meler et al. 2004). In addition, many studies have observed an attenuation of the photosynthetic response to elevated [CO_2] as N supply declines (Drake et al. 1997; Ainsworth and Long 2005). This study was consistent with these previous findings. The smaller stimulation of photoassimilate supply for respiration by elevated [CO_2] under limiting N supply compared to ample N supply provides a direct mechanism to explain the interactive effects of elevated [CO_2] and N supply on respiration. Under limited N supply, the smaller stimulation of A_{sat} by elevated [CO_2] could alter demand for energy from respiration in addition to varying substrate supply. Phloem loading can account for an estimated ~30% of nighttime energy demand (Bouma 1995). Since greater whole-plant growth at elevated [CO_2] can only result from stimulated photosynthesis if photoassimilate export from leaves is greater, this provides a potential explanation for variation in supply and demand control of respiration in response to interacting elevated [CO_2] and N supply.

The nature of altered demand at night for C skeletons and energy from leaf respiration when plants are grown at elevated [CO_2] is hard to assess. This study focused on mature leaves where respiration supplies "maintenance" processes, without the additional complication of growth processes found in developing leaves or whole-plant analyses. In addition to phloem loading, there are a number of significant sinks for respiratory products/energy in mature leaves, including protein turnover and maintenance of ion concentration and pH gradients (Penning de Vries et al. 1983; Cannell and Thornley; 2000). Protein turnover is estimated to account for approximately 20-30% of energy demand at night (Barneix et al. 1988; Bouma et al. 1994). It has been frequently asserted that lower leaf protein status, and thereby protein turnover, at elevated [CO_2] could exert a negative effect on demand for respiratory products and therefore suppress respiration rate (Ryan 1991; Amthor 1990; Bunce 1994; Poorter et al. 1997; Curtis and Wang 1998; Drake et al. 1999; Gonzalez-Meler et al. 2004). A reduction in leaf protein status in response to elevated [CO_2] is more likely as N supply decreases (Conroy and Hocking 1993; Drake et al. 1997; Taub and Wang 2008). Contrary to this expectation, leaf protein status responded to elevated [CO_2] equally under limiting N supply and ample N supply. There was no significant effect of elevated [CO_2] on protein content per unit leaf area, at either level of N

supply. Protein concentration per unit dry mass decreased at elevated [CO_2], possibly due to dilution by greater carbohydrate and cell wall contents (Teng et al. 2006; Taub and Wang 2008). Changes in N acquisition and allocation to protein could also have contributed to the effect (Taub and Wang 2008). However, if that did cause reduced demand for respiratory products at elevated [CO_2], it did so equally in ample N and limited N treatments – and therefore cannot have been the basis for a smaller stimulation of respiration by elevated [CO_2] under limiting N supply. In addition, the sucrose to amino acid ratio was greater in elevated [CO_2] and lower in ample N supply, but again there was no interaction effect of elevated [CO_2] and N supply. This ratio indicates that elevated [CO_2] perturbed the relative pool sizes of reduced carbon and reduced nitrogen available to biosynthetic pathways to similar degrees under ample N and limiting N supplies. The global transcriptional response to elevated [CO_2] also reinforces this notion because the identity of transcripts responding to the elevated [CO_2] treatment was similar under limiting N and ample N supplies. This is consistent with a common set of gene networks responding to signals associated with greater photoassimilate availability at both ample and limiting N supply.

The effect of elevated [CO_2] on leaf N concentration has been studied extensively. In plants grown at elevated [CO_2] with a limiting N supply, N concentration is typically reduced. If N is readily available, little or no change in N concentration occurs (Conroy and Hocking 1993; Weber et al. 1994; Lloyd and Farquhar 1996; Rogers et al. 1996a, b; Farage et al. 1998; Ainsworth and Long 2005; Taub et al. 2008a). The same pattern of response was observed in this study, but leaf N was not a consistently reliable proxy for leaf soluble protein content. Under limiting N supply, the impacts of elevated [CO_2] on leaf N and protein were consistent. However, under ample N supply, elevated [CO_2] led to a decrease in protein concentration proportional to changes in SLA while there was no change in N concentration. This would be consistent with plants grown with ample N supply at elevated [CO_2] being limited by the availability of an alternative nutrient and storing excess N as nitrate. Nitrate can represent 20% of the leaf N pool in herbaceous species (Millard 1988) and responds much more strongly than either protein or amino acid contents to N supply treatments similar to those used in this study (Tschoep et al. 2009).

While the concept that reduced leaf N may drive decreases in respiration at elevated [CO_2] has proven very popular in the literature, it is worth noting that there is not always a

significant relationship between leaf N concentration and respiration rate (Barneix et al. 1988; Amthor 1989; Byrd et al. 1992). Consequently, Gifford (2003) concluded that there was enough variability in the respiration-N relationship that the case was not strong for building mechanistic models for maintenance respiration based solely on N content. By comparison with N analyses, far fewer papers have directly assessed leaf soluble protein content responses to elevated [CO_2]. While reduced leaf protein content per unit leaf area has been observed in a number of cases (Rogers and Conroy 1996; Sicher and Bunce 1997; Rogers et al. 2002), there is clear precedent for the finding in this study of no change in protein content on an area or fresh weight basis (Isopp et al. 2000; Vu et al. 2002; Bae and Sicher 2004; Ainsworth et al. 2007). It is also important to point out that although N availability was limiting for growth, the limiting N treatment was not excessive enough to induce a severe N deficiency. This is supported by the N availability significantly changing ~1700 genes across the data set and similar leaf N concentrations as observed in another Arabidopsis N limitation study (Tschoep et al. 2009). This is in contrast to N starvation studies where nearly every functional category of genes significantly changed when N was resupplied (Schieble et al. 2004). Under such circumstances, the respiratory response to elevated [CO_2] might be very different. Therefore, further studies of this type, as well as attempts to quantify the impact of elevated [CO_2] on the demand for C skeletons and energy for protein turnover from respiration would be valuable.

The stimulation in leaf respiration at night was associated with greater abundance of transcript encoding respiratory genes including components of glycolysis, TCA cycle, mitochondrial electron transport chain, and mitochondrial import proteins (Figures 3.5 and 3.6). This response included greater transcript abundance for phosphofructokinase (Figure 3.5), which is generally considered the first committed step to the glycolytic pathway under non-stressful conditions (Plaxton 1996) and the enzymes catalyzing the CO_2 producing steps of the TCA cycle (Figure 3.6; Pyruvate Dehydrogenase complex; Isocitrate dehydrogenase; alpha-ketogluterate dehydrogenase; NADP-Malic Enzyme; Plaxton and Podesta 2006). While transcript abundance does not necessarily correlate with encoded protein abundance due to post-transcriptional and translation regulation, protein abundance for some mitochondrial proteins are highly correlated with transcript abundance across multiple tissue types (Lee et al. 2012). When comparing transcripts that were examined in the current study with Lee et al. (2012), transcripts that had significantly greater abundance under elevated [CO_2] for example Succinate dehydrogenase 1

(SDH1-1), translocase of the outer mitochondrial membrane 40 (TOM40-1), succinyl-CoA ligase, and aconitate hydratase 2 (ACO2) were shown by Lee et al. (2012) to have significantly high correlations ($r > 80$) with protein abundance. Interestingly, transcripts coding for fumerase (FUM1) and electron-transfer flavoprotein:ubiquinone oxidoruductase, which both had significantly reduced transcript abundance in elevated CO_2 in the current study, were shown to not be significantly correlated with protein levels (Lee et al. 2012).

Examining the transcriptional data set as a whole shows genes that have significantly greater abundance in elevated [CO_2] responded more in ample N versus limiting N during the midday time point, but the response to elevated [CO_2] was similar in ample and limiting N during the midnight time-point. Although the functional significance of this finding cannot currently be determined it is interesting in light of recent data demonstrating circadian control of transcriptional and enzymatic activity for primary and secondary metabolism (Graf et al. 2010; 2011; Kerwin et al. 2011) and the general transcriptional response to elevated [CO_2] being dependent on daylength (Queval et al. 2012). Furthermore, an experiment examining polysome loading, which is considered a good proxy for what transcripts are actively being translated into protein, provides evidence that a majority of Arabidopsis rosette protein turnover occurs during the light period when more energy is available compared to during the dark when growth and maintenance processes must be maintained on starch reserves alone (Piques et al. 2009). A stronger transcriptional response to elevated [CO_2] in the ample N treatment during the day may reflect that this is when the majority of protein synthesis takes place in Arabidopsis and would also diminish the significance of leaf protein status as a driver of leaf respiration responses to elevated [CO_2] at night.

Conclusions

This study demonstrates that the effect of elevated [CO_2] on leaf photosynthesis and respiration is attenuated by limiting N supply in Arabidopsis. There was no interaction between the effect of elevated [CO_2] and N supply on leaf protein status. Therefore, smaller stimulations of substrate supply and demand for energy from phloem loading by elevated [CO_2] appear to be the most parsimonious explanation for the attenuated respiratory response to elevated [CO_2] under limiting N versus ample N supply. Variation in N supply may therefore be an important contributing factor to the variable responses of respiration to elevated [CO_2] that have been previously reported. Future studies should be designed to reflect that the small relative and

absolute differences in leaf respiratory CO_2 fluxes between ambient and elevated [CO_2] observed in this study would be challenging to detect without the use of a custom-built gas exchange system. The finding of a conserved transcriptional response to elevated [CO_2] across soybean, rice, and Arabidopsis suggests that common regulatory mechanisms exist to control sink-source balance across diverse herbaceous species. Finally, the effects of elevated [CO_2] and N supply on transcript profiles were observed to be dependent on the time of day, demonstrating a commonality between the response of carbon metabolism to both feast and famine (Blasing et al. 2005; Usadel et al. 2008; Gibon et al. 2009; Queval et al. 2012).

Materials and Methods

Plant Growth Conditions

Arabidopsis thaliana (Col) seeds were surface sterilized with 70% ethanol solution for 2 minutes and a 15% Clorox solution for 15 minutes with occasional shaking, before being rinsed 5 times in sterile DI water. Seeds were plated on sterilized 0.5% gellan gum (Sigma, MO, USA) containing 0.5 x MS salts (Sigma, MO, USA) and 0.3% sucrose (pH 5.7) in a sterile hood where the plates were wrapped in aluminum foil and stored at 4 °C for 48 hours to synchronize emergence. Plates were removed from foil and placed in growth chambers vertically to allow for downward root growth. 5 days after emergence, seedlings were transplanted to 514 cm^3 pots containing LC1 Sunshine Mix (Sun Gro Horticulture, WA USA) mixed homogeneously with 20% v/v of small grain vermiculite. Two identical growth chambers (PGR14, Conviron, Winnepeg, Canada) were used to provide growing conditions of 10/14 hour day/night cycle at 21 °C/18 °C, 70% RH, and 250 μmol m^{-2}/s^{-1} of photosynthetically active radiation. Trays of 18 pots were rotated within chambers every other day to reduce in chamber variance in light levels and between chambers every five days to reduce any chamber bias. Pots were watered by adding 1L of 40% Long Ashton solution (Hewitt and Smith 1975) per tray once per week until week 4 when trays were watered every five days. NH_4NO_3 concentration was varied in the Long Ashton solution to establish the N treatments as limiting (0.25 mM NH_4NO_3) and ample (6 mM NH_4NO3). CO_2 concentration was maintained at either 370 ppm (ambient) or 750 ppm (elevated). With the exception of final biomass, which involved all aboveground tissue, the following analyses were preformed on the youngest most fully expanded leaves 35 days after germination.

Leaf Level Physiology

Photosynthetic CO_2 assimilation at growth CO_2 concentration, saturating light intensities (900 μmol m^{-2}/s^{-1} PPFD), and 21 °C was measured at dawn using a LI-6400 portable infrared gas analyzer (n=8; LICOR, Lincoln Nebraska, USA). In order to avoid significant measurement artifacts identified when using open-path gas analyzers to measure small respiratory fluxes of CO_2 (Jahnke 2001; Gifford 2003) midnight dark respiratory CO_2 efflux was measured using a custom designed closed gas exchange system (n=8) built around a LI-840 infrared gas analyzer (LICOR, Lincoln Nebraska, USA). The custom system consisted of an inline, DC brushless pump (Brailsford, NH) circulating air at 0.5 L min^{-1} to a leaf chamber (5.7 cm x 2 cm x 0.5 cm, L

x W x H). The chamber was custom machined out of aluminum with rounded corners to maximize chamber mixing. Nickel polytetrafluoroethylene (PTFE) (Teflon^{TM}) coating along with stainless steel tubing and fittings were used to minimize adsorption and absorption of water and CO_2 off of the internal surfaces of the system. The leaf chamber contained a thermocouple to monitor leaf temperature and a custom machined water jacket to control temperature from a circulating water bath. Whole plants were kept in the dark while attached leaves were sealed into the chamber around the base of the leaf blade by application of non-stick putty (Qubitac sealant; Qubit Systems, Kingston, Canada). An O-ring between the chamber base and lid was sealed by pressure from two spring-loaded clips. A two-way valve was used inline on the system to vent excess CO_2 while the leaves were equilibrating to the chamber (1-2 minutes) and leaf temperature was stable at 18 °C. Once measurements commenced, the system was completely sealed and CO_2 concentration increase over time was recorded through a CR1000 datalogger (Campbell Scientific; Logan UT, USA). After 1 minute of recording linear CO_2 increase (At least a 50 ppm rise in [CO_2] from the start of the measurement) the chamber was opened, the leaf was excised and photographed for leaf area. Leaf area was determined using Image J (http://rsbweb.nih.gov/ij/). CO_2 increase per unit time was calculated for each replicate using a linear regression model (PROC REG; SAS, Cary NC, USA) and rates were corrected for leaf area. Five of these independent respiration systems running simultaneously allowed for measurement of ~25 individuals in one hour. Rates were measured at subjective midnight as preliminary data collected demonstrated that the middle 4 hours of the dark period to had the greatest and most stable R (Figure 3.7).

Gene Expression

Youngest most fully expanded leaves were excised from individual replicate plants (n=3-5) at midday and midnight on day 35, wrapped in aluminum foil, immediately plunged into liquid N_2, and stored at -80°C until total RNA was isolated using a Spectra Plant RNA Isolation Kit (Sigma, St. Louis MO, USA) following manufacturer's instructions. The cRNA labeling, and subsequent steps leading to hybridization and scanning of the Genechip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara CA, USA) were performed by the Keck Center for Comparative and Functional Genomics at the University of Illinois (www.biotech.uiuc.edu/centers/Keck) following manufacture's protocols.

Leaf carbohydrates, soluble protein, and amino acids

Leaf disks (1.2 cm^2) were collected from the youngest most fully expanded leaves at midnight on day 35 (n=8), wrapped in aluminum foil, and immediately plunged into liquid N, and stored at -80°C until carbohydrates, protein, and amino acids were extracted and analyzed as described by Ainsworth et al. (2007).

Specific Leaf Area, Leaf N content and Biomass

Leaves excised after respiration measurements were oven dried at 70°C and weighed (n=8). Subsequently, the dried leaf material was powdered and analyzed for N content using an elemental combustion system (Model 4010; Costech Analytical Technologies, Valencia, CA, USA) as described by Leakey et al. (2006). 35 DAE whole plants of each treatment were excised at the soil surface, oven dried at 70°C, and weighed for final above ground biomass.

Statistics

All leaf physiological and biochemical parameters were tested using an ANOVA (PROC GLM, SAS 9.1; SAS, Cary NC, USA). In all tests, CO_2 and nitrogen treatments were considered fixed effects and a p-value <0.05 was the threshold for significance. Following the detailed protocols of Leakey et al. (2009a) for microarray analysis, the transcriptional data set was analyzed using an ANOVA (JMP Genomics 5.1; SAS, Cary NC, USA). In-brief, CO_2, nitrogen (N) and time of day (TOD) were each considered fixed effects in the model. Individual transcripts were not tested if they were not present in at least three of the replicated chips for each CO_2xNxTOD treatment combination. Hierarchical clustering analysis was performed on all transcripts that were differentially expressed in one or more of the treatment combinations ($p < 0.05$) using the Ward clustering method option in JMP Genomics 5.1. Regression analysis for genes responding significantly to elevated [CO_2] in each level of N were performed (PROC REG; SAS, Cary NC, USA).

Tables and Figures

Table 3.1 Number of transcripts responding significantly ($p < 0.05$) to each of the main effects and/or interactions in the ANOVA model of the 12,826 genes tested in at least three biological replicates.

Factor in ANOVA model	Number of Significant Transcripts
CO_2 (C)	4439
Nitrogen (N)	1708
Time of Day (TOD)	8640
C x N	258
C x TOD	678
N x TOD	812
C x N x TOD	376

Table 3.2 List of transcripts that were significant for the main effect of elevated [CO_2] that are displayed in Figures 3.5 and 3.6. AT locus IDs, functional description, and percent change in gene expression in elevated [CO_2] versus ambient [CO_2], with a negative percentage indicating a greater expression in ambient.

Fig.	AT Number	Description	Ample N Day	Ample N Night	Limiting N Day	Limiting N Night
5	(At1g22650)	beta-fructofuranosidase,	49.78467	47.812675	32.490047	40.95552
5	(At1g56560)	beta-fructofuranosidase,	-14.081125	-5.5465107	-2.3495905	-19.523302
5	(At5g22510)	beta-fructofuranosidase,	-9.468862	4.9446774	-7.21471	-22.98726
5	(At1g35580)	CINV1 | CINV1 (cytosolic invertase 1);	28.023176	14.92431	5.7343397	17.769176
5	(At5g37180)	SUS5, ATSUS5 | SUS5; UDP-glycosyltransferase/ sucrose synthase	-25.437094	-27.29432	-27.022179	-42.211273
5	(At1g73370)	SUS6, ATSUS6 | SUS6 (SUCROSE SYNTHASE);	-26.518143	-14.063079	-25.044825	-45.257244
5	(At5g17310)	UTP--glucose-1-phosphate uridylyltransferase, putative	-20.53811	-46.740417	-27.526674	-14.439014
5	(At2g19860)	ATHXK2, HXK2 | HXK2 (HEXOKINASE 2);	-10.44909	-24.003546	-1.6529965	-37.996395
5	(At1g70730)	phosphoglucomutase, cytoplasmic, putative	-13.307085	-19.861578	-9.601546	-21.087542
5	(At4g26270)	PFK3 | PFK3 (PHOSPHOFRUCTOKINASE 3);	139.81468	11.129897	91.92598	45.290966
5	(At5g56630)	PFK7 | PFK7 (PHOSPHOFRUCTOKINASE 7);	7.4418464	7.5771	19.921057	7.0486794
5	(At2g36460)	fructose-bisphosphate aldolase, putative | chr2	11.870139	33.25226	9.041552	8.031347
5	(At4g26520)	fructose-bisphosphate aldolase, cytoplasmic | chr4	-20.23764	-27.843563	-26.836296	-35.03174
5	(At3g04120)	GAPC, GAPC-1, GAPC1 | GAPC1 (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE)	7.175489	58.29306	-1.7861085	44.43256
5	(At1g08940)	phosphoglycerate/bisphosphoglycerate mutase family protein | chr1	26.751253	65.77196	37.21327	61.19869
5	(At1g74030)	enolase, putative | chr1	-20.81989	-25.692486	-12.861028	-10.8776
5	(At5g63680)	pyruvate kinase, putative | chr5	40.709137	37.466682	35.528755	49.37917
5	(At2g42600)	ATPPC2 | ATPPC2 (PHOSPHOENOLPYRUVATE CARBOXYLASE)	-18.504404	-14.91982	8.122817	-28.706572
5	(At1g53310)	ATPPC1 | ATPPC1 (PHOSPHOENOLPYRUVATE CARBOXYLASE)	23.17069	16.222952	33.141838	27.216871
5	(At3g14940)	ATPPC3 | ATPPC3 (PHOSPHOENOLPYRUVATE CARBOXYLASE)	-33.57538	-11.907567	-28.779226	-16.921907
6	(At2g05710)	aconitate hydratase	14.0621395	10.186082	28.493378	1.7841556
6	(At4g26970)	aconitate hydratase	-4.8293257	30.927576	0.6707104	12.85247
6	(At4g35260)	IDH1 | IDH1 (ISOCITRATE DEHYDROGENASE 1)	9.59652	28.434786	12.566458	14.817377
6	(At2g17130)	IDH2 | IDH2 (ISOCITRATE DEHYDROGENASE SUBUNIT 2)	43.50679	44.09797	17.86889	25.421267
6	(At5g55070)	2-oxoacid dehydrogenase family protein	9.448258	24.902382	-1.0855637	31.685364
6	(At5g08300)	succinyl-CoA ligase (GDP-forming) alpha-chain	30.230106	7.679081	8.930881	17.729265
6	(At5g66760)	succinate dehydrogenase 1|	13.27983	20.256344	0.56853515	15.792916
6	(At5g40650)	SDH2-2 | SDH2-2; electron carrier/ succinate dehydrogenase | chr5	7.090908	40.991188	15.26512	28.885414
6	(At3g27380)	SDH2-1 | SDH2-1; electron carrier/ succinate dehydrogenase | chr3	10.571456	45.706676	20.776436	21.864592
6	(At2g47510)	FUM1 | FUM1 (FUMARASE 1); catalytic/ fumarate hydratase | chr2	-8.915656	-7.6455107	-5.8863425	-37.543526
6	(At1g79750)	ATNADP-ME4 | ATNADP-ME4 (NADP-malic	-10.625758	-5.1877747	-12.343316	-12.447031

		Table 3.2 (cont.)				
Fig.	AT Number	Description	Ample N Day	Ample N Night	Limiting N Day	Limiting N Night
6	(At1g16700)	NADH-ubiquinone oxidoreductase	15.239156	5.400302	12.56638	8.765047
6	(At5g08740)	NDC1 | NDC1 (NAD(P)H dehydrogenase C1); NADH dehydrogenase | chr5	-3.8417864	-9.318133	-15.689513	-25.247278
6	(At2g29990)	NDA2 | NDA2 (ALTERNATIVE NAD(P)H DEHYDROGENASE 2);	57.87875	74.99589	62.513435	80.98047
6	(At1g07180)	ATNDI1, NDA1 | NDA1 (ALTERNATIVE NAD(P)H DEHYDROGENASE 1);	-36.97374	-4.8536644	-22.477339	-21.725733
6	(At4g28220)	NDB1 | NDB1 (NAD(P)H dehydrogenase B1);	-4.004412	-23.694286	-15.117091	-26.052412
6	(At4g05020)	NDB2 | NDB2 (NAD(P)H dehydrogenase B2);	25.258717	145.71263	25.37911	177.83879
6	(At1g50940)	ETFALPHA | ETFALPHA (electron transfer flavoprotein alpha);	14.306861	21.615238	22.298439	6.723139
6	(At2g43400)	ETFQO | ETFQO (electron-transfer flavoprotein	-7.549053	-12.193479	-19.209364	-12.56517
6	(At5g25450)	ubiquinol-cytochrome C reductase complex 14 kDa protein, putative | chr5	25.655167	60.102962	26.979887	184.06479
6	(At3g51790)	ATG1 | ATG1 (ARABIDOPSIS TRANSMEMBRANE PROTEIN G1P-RELATED 1) | chr3	26.245329	16.569471	8.967658	37.306507
6	(At1g49380)	cytochrome c biogenesis protein family | chr1	-30.230326	-33.697784	-33.20338	-23.078436
6	(At4g39740)	electron transport SCO1/SenC family protein | chr4	42.52253	8.718692	31.529827	26.594086
6	(At1g69750)	COX19-2, ATCOX19-2 | cox19 family protein | chr1	25.376677	11.071525	32.87171	10.56487
6	(At5g04750)	F1F0-ATPase inhibitor protein, putative | chr5	26.80846	19.218903	24.133284	38.21546
6	(At5g12420)	unknown protein | chr5	110.73355	1.7338649	-9.386997	70.97791
6	(At5g49460)	ACLB-2 | ACLB-2 (ATP CITRATE LYASE SUBUNIT B 2);	-5.1883664	-21.864883	-13.668199	-24.407814
6	(At1g10670)	ACLA-1 | ACLA-1; ATP citrate synthase | chr1	-3.0739744	-14.689158	-4.9259186	-22.98726
6	(At3g06650)	ACLB-1 | ACLB-1; ATP citrate synthase | chr3	-13.862761	-22.444553	-20.726147	-9.248965
6	(At1g30120)	PDH-E1 BETA | PDH-E1 BETA (PYRUVATE DEHYDROGENASE E1 BETA);	-6.8370647	-7.670537	-8.895135	-25.322994
6	(At1g01090)	PDH-E1 ALPHA | PDH-E1 ALPHA (PYRUVATE DEHYDROGENASE E1 ALPHA);	-16.522402	-16.584986	-10.786929	-22.253965
6	(At3g52200)	LTA3 | LTA3; ATP binding / dihydrolipoyllysine-residue acetyltransferase | chr3	10.204185	9.784015	6.27255	5.3058076
6	(At1g34430)	EMB3003 | EMB3003 (embryo defective 3003); acyltransferase/	-11.268701	-29.313236	-22.293457	-27.771555
6	(At3g13930)	dihydrolipoamide S-acetyltransferase, putative | chr3	-11.787745	-11.032704	-14.047233	-11.583471
6	(At1g48030)	mtLPD1 | mtLPD1 (mitochondrial lipoamide dehydrogenase 1);	-10.575317	-25.631994	-16.11644	-30.822058
6	(At3g16950)	LPD1, ptlpd1 | LPD1 (LIPOAMIDE DEHYDROGENASE 1);	-14.211867	-35.808685	-17.747011	-41.8577
6	(At3g17240)	mtLPD2 | mtLPD2 (LIPOAMIDE DEHYDROGENASE 2);	20.490633	23.356352	23.672009	2.5448472
6	(At1g04070)	TOM22-I, ATTOM22-I | TOM22-I (TRANSLOCASE OF OUTER MEMBRANE 22-I);	32.962555	16.885826	-6.1408043	36.74992
6	(At1g27390)	TOM20-2 | TOM20-2 (TRANSLOCASE OUTER MEMBRANE 20-2);	19.684805	36.11452	-2.5144699	21.78226
6	(At3g25120)	mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23	23.923346	16.569551	7.1574383	21.30895
6	(At5g63680)	pyruvate kinase, putative | chr5	40.709137	37.466682	35.528755	49.37917

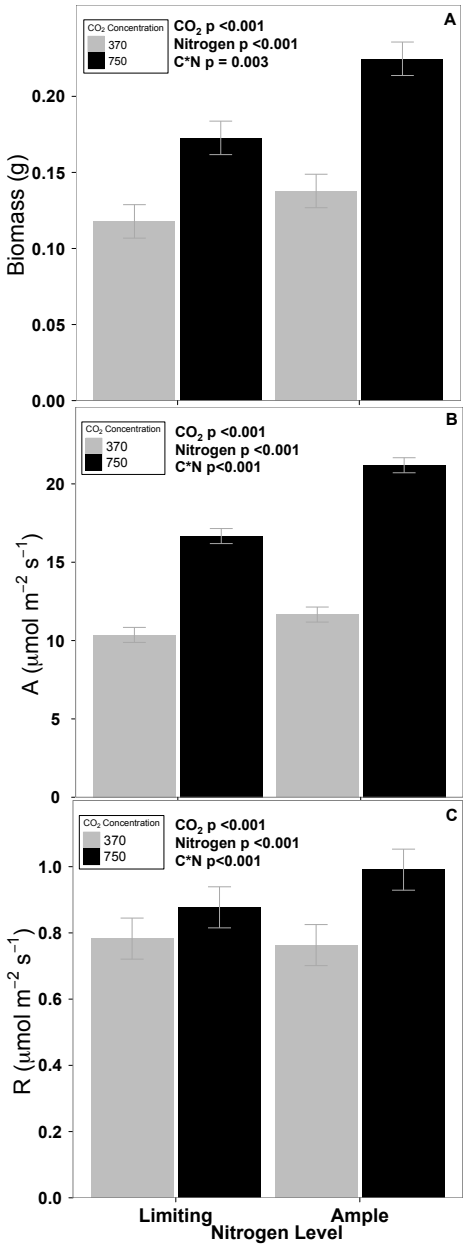

Figure 3.1 Panels are total above ground dry biomass (A), light saturated CO_2 assimilation (A) at growth [CO_2] (B), and dark respiration (R) rates taken at subjective mid-night (C). Mean values (+/- standard errors) of physiological parameters of plants growing in ambient (370 ppm) or elevated (750 ppm) [CO_2] and limiting or ample N conditions. Also plotted are the p-values from the statistical model each of the parameters.

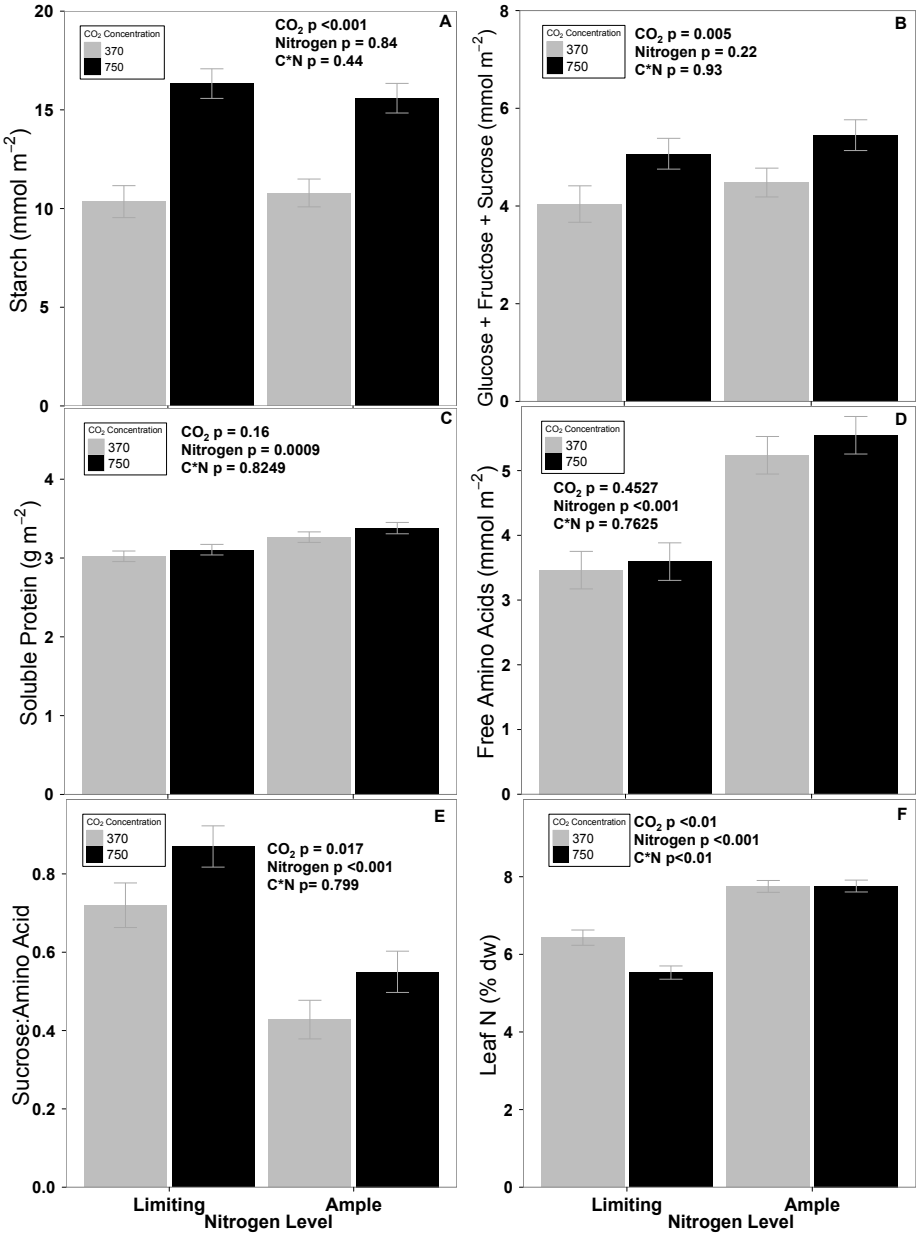

Figure 3.2 Panels are starch content (A), combined glucose, fructose, sucrose content (B), soluble protein content (C), free amino acids (D), Sucrose to Amino Acid Ratio (E), leaf N concentration percentage (F). Mean values (+/- standard errors) of biochemical parameters in the youngest most fully expanded leaves growing in ambient (370 ppm) or elevated (750 ppm) [CO_2] and limiting or ample N conditions collected at subjective midnight. Also plotted are the p-values from the statistical model for each of the parameters.

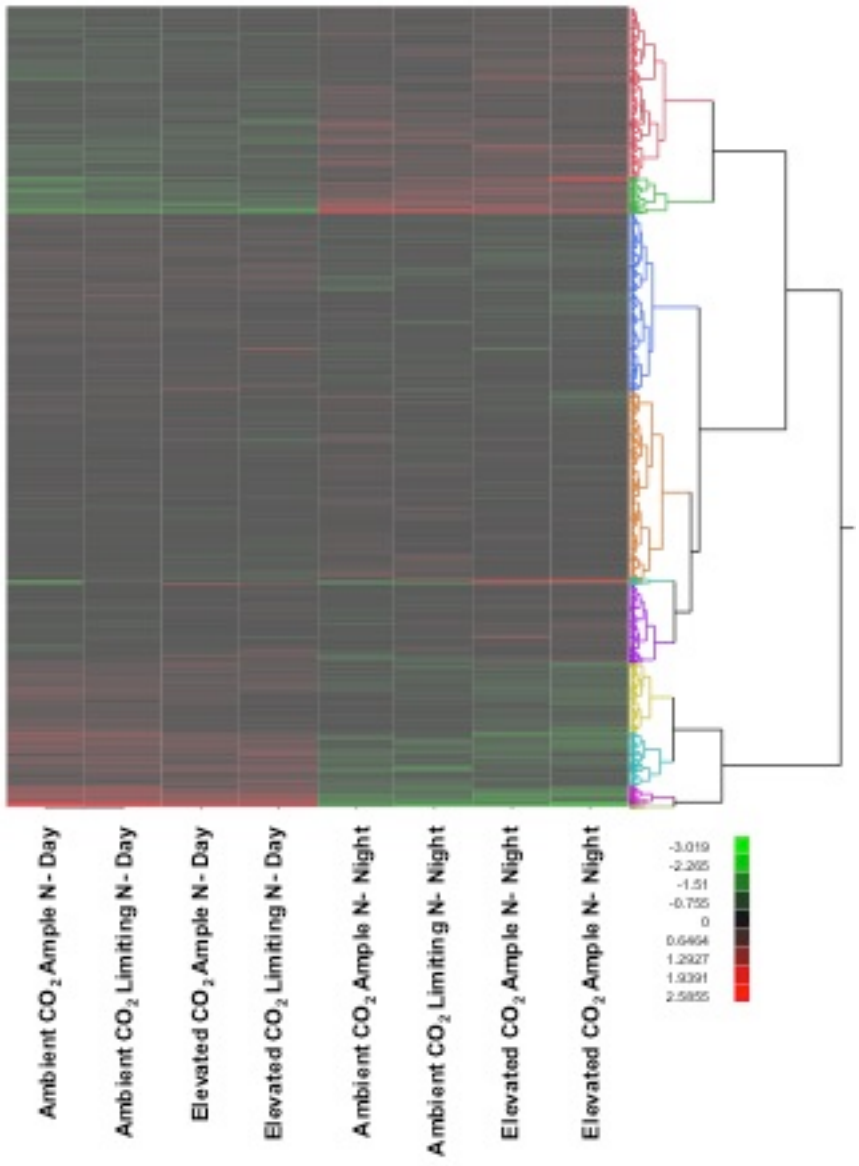

Figure 3.3 Hierarchical clustering of the normalized mean fluorescence intensity values (Log_2) of all significantly responding transcripts to at least one factor in the ANOVA model where, in addition to the main effects and interactions of CO_2 and N availability, time of day was also a fixed factor. Green indicates lower intensity and red indicates greater intensity compared to the mean intensity values (black). Tissue was harvested at midday (Day) and midnight (Night) from the same leaf cohort as the other leaf biochemical and physiological parameters.

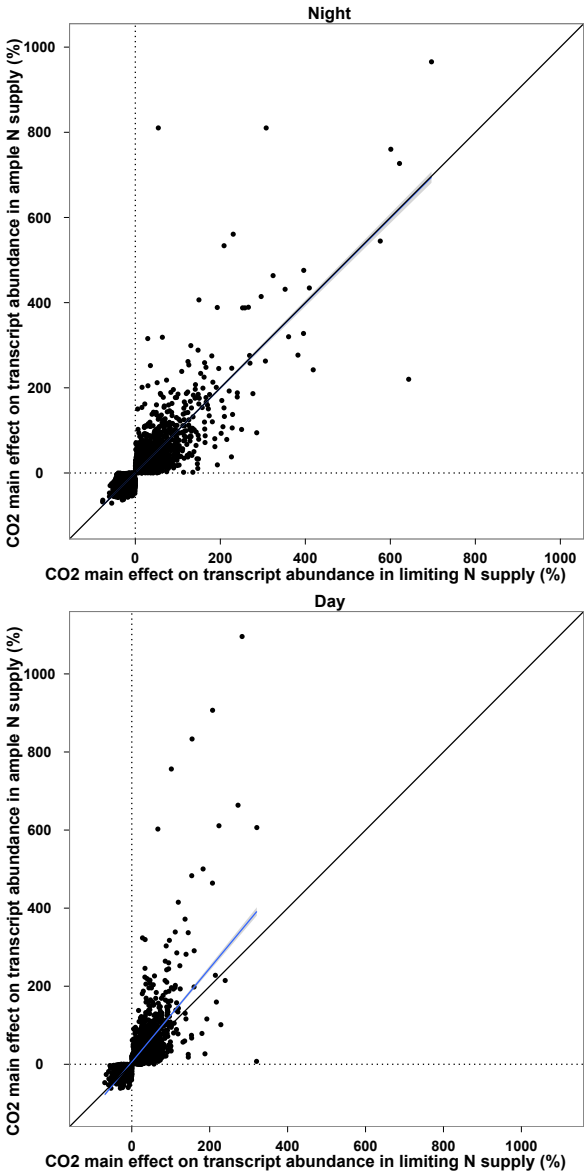

Figure 3.4 A regression plot of the relationship of genes responding significantly and in the same direction to elevated [CO_2] in limiting (x-axis) or ample (y-axis) N supply during mid-night (top) and mid-day (bottom). The blue line is the line of best fit with grey 95% confidence intervals while the black line is a 1:1 line.

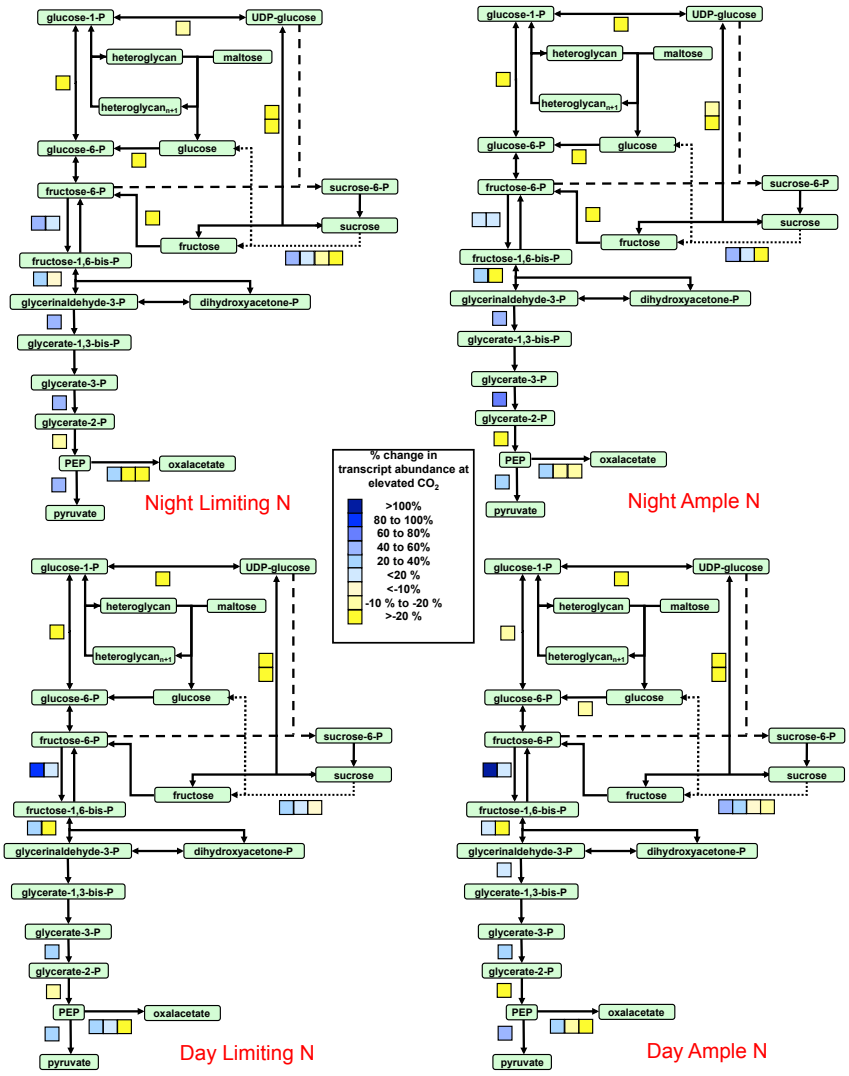

Figure 3.5 Graphical representation of genes encoding components of sugar transformations reactions and glycolysis that responded to elevated [CO_2] during midnight (top) or midday (bottom) and limiting N (left) or ample N (right). Each blue (positive percentage change) and yellow (negative percentage change) represents the mean value of a unique transcript that responded significantly ($P < 0.05$) to elevated [CO_2]. Details about individual transcripts can be found in Table 3.2.

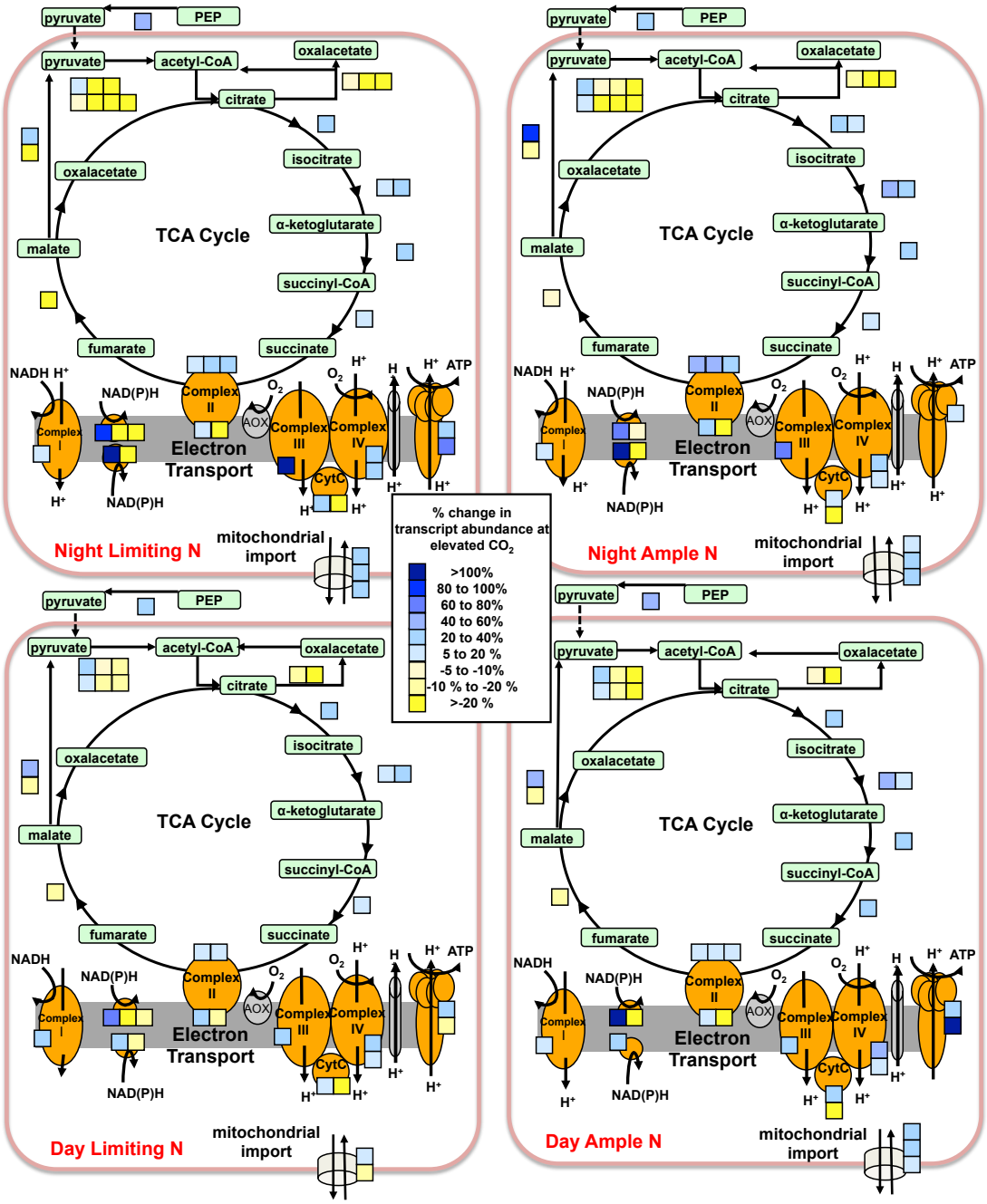

Figure 3.6 Graphical representation of genes encoding components of the TCA cycle and mitochondrial electron transport chain that responded to elevated [CO_2] during midnight (top) or midday (bottom) and limiting N (left) or ample N (right). Each blue (positive percentage change) and yellow (negative percentage change) represents the mean value of a unique transcript that responded significantly ($P < 0.05$) to elevated [CO_2]. Details about individual transcripts can be found in Table 3.2.

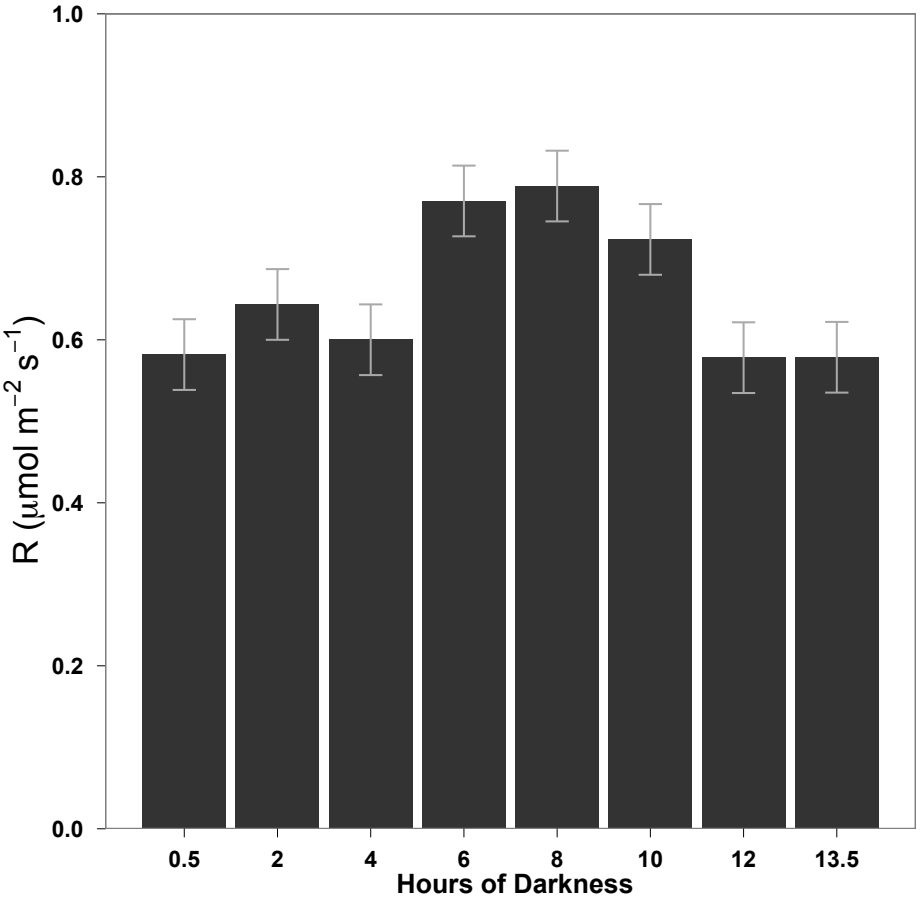

Figure 3.7 Mean values (+/- standard errors) of dark respiration rates of youngest most fully expanded leaves of plants grown in ambient [CO_2] and ample N supply during the dark period starting 0.5 hours after the onset of dark and continuing every two hours of the 14 hour dark period.

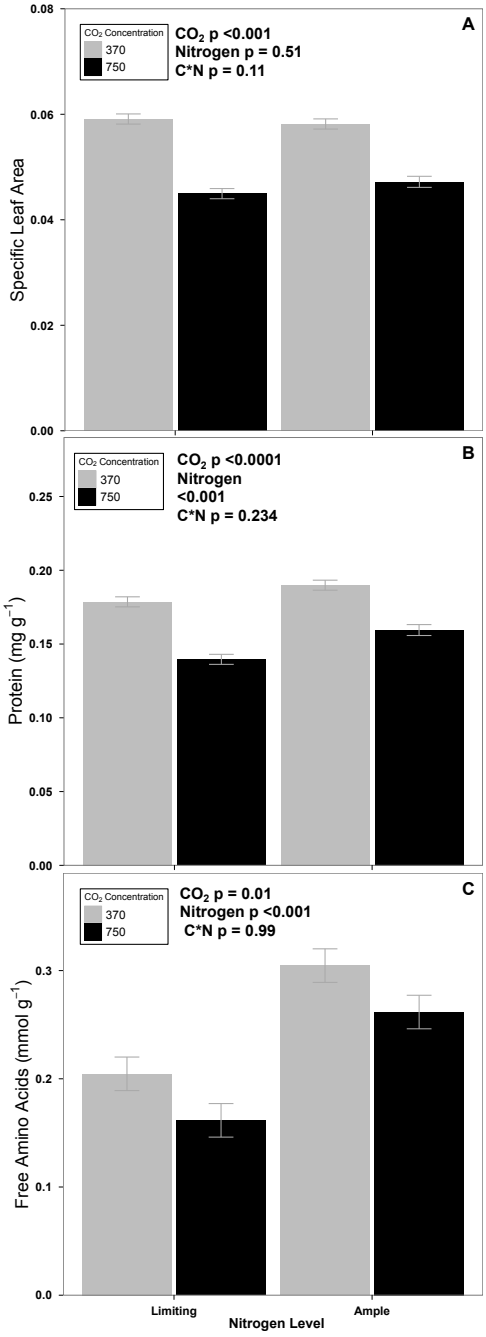

Figure 3.8 Mean values (+/- standard errors) of specific leaf area (SLA), leaf protein (mass basis), and amino acids (mass basis) of fully expanded leaves grown in ambient (370 ppm) or elevated (750 ppm) [CO_2] and limiting or ample N conditions.

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CHAPTER IV: ELEVATED [CO2] INDUCED TRANSCRIPTIONAL REPROGRAMMING OF RESPIRATION AND A STIMULATION OF DARK RESPIRATION AS ARABIDOPSIS THALIANA LEAVES TRANSITION FROM SINKS TO SOURCES

Abstract

A mechanistic understanding of respiration is critical to elucidating the relationship between increased atmospheric CO_2 concentration ($[CO_2]$) and plant function because this process provides the carbon skeletons and energy needed for growth and maintenance. Greater abundance of transcripts encoding the respiratory pathway (glycolysis, TCA cycle, mitochondrial electron transport) have been observed in mature Arabidopsis, rice and soybean leaves grown at elevated $[CO_2]$. This suggests transcriptional reprogramming of metabolism underpins greater rates of dark respiration and greater mitochondrial numbers in leaves of plants grown at elevated $[CO_2]$. In addition, within hours of mature Arabidopsis leaves being exposed to elevated $[CO_2]$, changes in gene expression in younger expanding leaves which were not exposed to the elevated $[CO_2]$ treatment have been observed, suggesting systemic signaling. This study tested the hypothesis that elevated $[CO_2]$ induces transcriptional reprogramming and stimulation of respiration throughout leaf development starting with the primordia and continuing through key phases of cell division and leaf expansion. Beginning in early leaf expansion, elevated $[CO_2]$ increased glucose concentration and caused transcriptional reprogramming of respiration. These effects occurred prior to detectable starch accumulation or treatment effects on sucrose concentration. Stimulation of dark respiration from elevated $[CO_2]$ was only distinguishable as leaves transitioned from rapidly expanding (sink) tissues without starch storage to mature (source) tissues that stored more starch in the elevated $[CO_2]$ treatment. These results suggest that the transition between expanding tissue that must support both growth and maintenance processes to mature tissue that only has maintenance respiration requirements is a key developmental stage that defines the difference in respiration rates between ambient and elevated $[CO_2]$ in mature tissues.

Introduction

Atmospheric CO_2 concentration ($[CO_2]$) has been increasing since the beginning of the industrial revolution due to anthropogenic CO_2 emissions from the combustion of fossil fuels and land use change (Canadell et al. 2007; Friedlingstein et al. 2010). Elevated atmospheric $[CO_2]$ generally increases photosynthetic CO_2 uptake in C_3 plants causing stimulations in plant biomass and yield (Ainsworth and Long 2005). However, a mechanistic understanding of respiration is critical to elucidating the relationship between increased atmospheric $[CO_2]$ and plant carbon balance and growth because this process provides the carbon skeletons and energy needed for growth and maintenance during the dark cycle, and at the same time releases CO_2 (Atkin et al. 2010). While many studies have examined the physiological and molecular photosynthetic responses to elevated $[CO_2]$ (Moore et al. 1999), far fewer have examined the respiratory response in the post-genomics era (Ainsworth et al. 2006; Leakey et al. 2009; Fukayama et al. 2011; Chapter 3). Transcriptional reprogramming of genes coding for respiratory machinery in response to elevated $[CO_2]$ in mature leaves has been observed across three functional groups of C_3 herbaceous species including a dicot (Arabidopsis; Chapter 3), a monocot (rice; Fukayama et al. 2011) and a legume (soybean; Ainsworth et al. 2006; Leakey et al. 2009). This suggests that a conserved respiratory response to a consistent stimulation of carbon availability. The transcriptional up-regulation of the respiratory pathway in elevated $[CO_2]$ treatments coincided with greater rates of leaf dark respiration in both Arabidopsis and soybean (Leakey et al. 2009; Chapter 3). These transcriptional and physiological responses are logically associated with the observation of greater mitochondrial numbers per cell in mature leaves of plants grown in elevated $[CO_2]$ across a large number of C_3 species (Griffin et al. 2001). In Arabidopsis, transcript abundance for components of the respiratory pathway, along with numbers of mitochondria per mesophyll cell, increase as cells expand and leaves mature (Skirycz et al. 2010; Preutin et al. 2010; Carrie et al. 2012). Yet it remains unclear if transcriptional modifications in elevated $[CO_2]$ are necessary early in leaf development in order to increase overall energy metabolism observed in mature leaves and how these modifications are coordinated with the general leaf developmental program.

Since the initial discovery that rising $[CO_2]$ reduced stomatal number on leaf surfaces and altered epidermal patterning, subsequent work has refined ideas about the molecular mechanisms

underpinning epidermal patterning in response to elevated [CO_2] across leaf development (Woodward 1987; Lake et al. 2001; Levine et al. 2009). Although much research has focused on these epidermal changes induced by differing CO_2 concentration, far fewer studies have examined the effects of elevated [CO_2] on metabolic machinery over the course of leaf development (Robertson et al. 1998a, b; Ainsworth et al. 2006). Within hours of mature Arabidopsis leaves being exposed to elevated [CO_2], changes in gene expression in younger expanding leaves which were not exposed to the elevated [CO_2] treatment have been observed, suggesting systemic signaling between mature and developing tissues (Coupe et al. 2006). Sugar levels increased in developing leaves within 2 hours of an elevated [CO_2] treatment being applied to mature leaves, and conversely, sugar levels decreased in developing leaves when mature leaves were shaded, implying that carbohydrate status is an important component for the systemic signaling of changes in plant carbon availability (Coupe et al. 2006). In support of this theory, increased numbers of mitochondria and chloroplasts per cell were observed in wheat leaves growing in elevated [CO_2] and the difference could be detected as early as twelve hours post mitosis in basal cells (Robertson and Leech 1995; Robertson et al. 1995). However, the transcriptional responses associated with such cellular restructuring are not known.

A well-described metabolic transition in developing leaves is from sink to source where leaves shift from being net importers of photoassimilate to net exporters (Ho 1988; Turgeon 1989). Source-sink relationships have been shown to be very important to the overall plant growth response to elevated [CO_2] (Rogers et al. 1996; Isopp et al. 2000;). Links between expression of key photosynthetic genes, chloroplast development and a gradual increase in photosynthetic rate as leaves mature and become source tissues have been described in both ambient and elevated [CO_2] grown plants (Ainsworth et al. 2006). If systemic signaling is occurring from mature leaves to developing leaves and important metabolic shifts are occurring during the source-sink transition, then examining changes in respiratory gene expression beginning with leaf primordia through the sink-source transition into mature tissue would be an important step forward to uncovering the mechanism behind altered respiratory metabolism in elevated [CO_2].

Arabidopsis is amenable for asking questions regarding the interaction between leaf development and elevated [CO_2] due to the existence of detailed vegetative developmental

timelines based on its simple rosette morphology (Boyes et al. 2001) and many reverse genetic studies allowing a functional examination of development and transcriptional networks in the absence of regulatory genes (Moore et al. 2003; Jiang and Deyholos 2009; Woo et al. 2010; Besseau and Palva 2012; Osnato et al. 2012). The respiratory demands on growing tissue are much greater to provide the carbon skeletons and reducing energy needed for both building and maintaining tissue instead of just maintenance respiration in mature tissue (Lambers et al. 2008). Our study is a time-course experiment in Arabidopsis that followed a leaf cohort across three key stages of leaf development (primordia, expansion, and mature) in ambient and elevated [CO_2]. These time points were specifically chosen as both key stages in leaf development and as important stages as leaves transition from net energy importers to net energy exporters. By integrating physiological, biochemical, and transcriptional data we specifically tested the hypothesis that elevated [CO_2] would induce transcriptional reprogramming and a stimulation of respiration during all three key stages in leaf development beginning with the primordia and continuing through leaf expansion to maturity. The rich transcriptional data generated in this study allowed us to ask additional *a-postori* questions regarding whether or not fundamental shifts in gene expression occur in the leaf developmental program in elevated [CO_2].

Results

Biomass, Photosynthesis, Respiration and Leaf Biochemistry

Elevated [CO_2] significantly stimulated plant biomass beginning at the expanding leaf time point (23 DAG; 10% stimulation), and the difference between treatments became greater when the plants moved into the exponential growth phase and leaf 10 was mature (30 DAG; 29% stimulation; Figure 4.1b). During the expanding leaf time-point (23 DAG), there was a significant stimulation in glucose content (42%) in the elevated [CO_2] treatment, but there was no significant difference in sucrose content (Figure 4.2). Additionally, there were no detectable levels of starch in either [CO_2] treatment during the expanding time point and there were no differences between [CO_2] treatments (Figure 4.2). The stimulation in final biomass was driven by greater photosynthetic carbon assimilation in mature leaves for example 30 DAG (+69%; Figure 4.1a). The stimulation of photosynthesis in elevated [CO_2] during the day in mature leaves lead to significantly greater starch (+90%), glucose (+107%), and sucrose (76%) content at midnight at the mature leaf time-point (30 DAG; Figure 4.2). These greater levels of non-

structural carbohydrates contributed to the significant 10% reduction in specific leaf area in the mature leaves (Figure 4.2 G). Leaf dark respiration rates varied significantly across development where rates were greater for rapidly expanding tissue (23 DAE) with a gradual decline as the leaves slowed expansive growth and transitioned into maturity (29, 30, and 31 DAE; Figure 4.3). When leaves were rapidly expanding there was no difference in dark respiration rates between ambient and elevated [CO_2] (23 DAG; Figure 4.3), but there was a significant stimulation of respiration in the elevated [CO_2] treatment from 24 DAG (+13%) onwards to 29, 30, and 31 DAG (+20-25%; Figure 4.3).

Transcript Profiles

The Arabidopsis chip used to analyze gene expression represented 24,000 genes. Of the 12,570 transcripts that were present in at least three3 samples from every [CO_2] by developmental stage treatment combination, nearly all the genes tested (11,337) were differentially expressed across the three leaf developmental time points ($p<0.05$), 2,137 were differentially expressed between ambient and elevated [CO_2], and 1,694 had a significant [CO_2] by developmental stage interaction (Table 4.1). Hierarchical-clustering of normalized intensity values for each treatment across the entire data set demonstrated that each developmental stage clustered together and that the differences in expression patterns between ambient and elevated [CO_2] were much smaller than the differences among developmental stages (Figure 4.4). Hierarchical-clustering of relative expression changes for developmental time points revealed that expression patterns across leaf development clustered into 10 general patterns (Figure 4.5; Table 4.2). Transcripts that were significant for the main effect of [CO_2] and the interaction between [CO_2] and developmental stage were represented in each of the 10 clusters, but the relative distribution of the significant transcripts between clusters was skewed. Sixty-two percent of the transcripts significant for a main effect of [CO_2] were part of clusters 7 through 10, which have a general pattern of increasing through leaf development (clusters 7 and 8) or peaking during the expanding time point (clusters 9 and 10; Figure 4.5; Table 4.2). Forty-one percent of the transcripts significant for a [CO_2] by developmental stage interaction were part of clusters 5, and 10 where cluster 5 genes gradually decreased in expression across development and cluster 10 genes peaked in expression during the expanding time-point (Figure 4.5; Table 4.2). Examining these patterns further by comparing MAPMAN functional categorization of

transcripts and their distributions between these clusters reveals that greater than 60% of the genes mapped to photosynthesis, major and minor carbohydrate metabolism, glycolysis, and the TCA cycle functional categories were part of clusters 7 through 10 (Figure 4.5; Table 4.2). The mitochondrial electron transport chain on the other hand, had only 38% of the genes represented in clusters 7 through 10 with 25% in clusters 5 through 6 and the rest being distributed over the remaining clusters (Figure 4.5; Table 4.2). Nearly all of the transcripts tested that coded for components of glycolysis, the TCA cycle and the mitochondrial electron transport chain had a main effect of development and were in the same clusters with all the transcripts having a main effect for [CO_2] or a [CO_2] by development interaction (Figures 4.6 and 4.7). Mapping the percentage change of transcripts coding for transcripts in elevated [CO_2] versus ambient [CO_2] onto metabolic pathways revealed that the relative difference between ambient and elevated [CO_2] gradually increases as leaves develop (Figures 4.5 and 4.6; Table 4.3).

Discussion

The hypothesis that transcriptional reprogramming of respiration and greater respiration rates would occur in the elevated [CO_2] treatment during the primordia, expanding and mature time-points was not fully supported. The difference in respiration and absolute differences of respiratory gene expression in elevated [CO_2] gradually became greater as leaves matured. Under these growth conditions, the expanding time point (23 DAG) is the first day, where leaf 10 is large enough that respiration can be accurately quantified using our custom designed system. While respiration rates were significantly greater in the rapidly expanding tissues at 23 DAG compared to mature tissues at 30 DAG, there were no significant differences between ambient and elevated [CO_2] during the expanding time point (Figure 4.2). Respiration rates are generally greater in rapidly expanding tissues because demands for energy and carbon skeletons are needed for both growth and maintenance processes instead of just maintenance in mature tissues (Lambers et al. 2008). At the expanding time point there were no detectable levels of starch in either [CO_2] treatment compared to the 90% stimulation in starch in the elevated [CO_2] treatment in mature tissues. The lack of starch pools and relatively high levels of mobile sugars indicate that expanding leaf 10 was a sink tissue at 23 DAG (Ho 1989; Turgeon 1989) with a stimulation in dark respiration occurring in elevated [CO_2] after leaf 10 started transitioning from sink to source (Figure 4.2). The significant stimulation of photosynthetic [CO_2] assimilation in mature

leaf 10 at 30 DAG caused a significant increase in starch, glucose and sucrose concentrations at night. The stimulation of photosynthesis leading to greater starch and carbohydrates at night is well documented (Reviewed in Ainsworth and Long 2005; Leakey et al. 2009b). Increased respiratory substrate availability in elevated [CO_2] led to increases in dark respiration at 30 and 31 DAG. The constant stimulation of photosynthetic carbon gain averaged across the rosette in elevated [CO_2] is the main upstream driver behind the increase in biomass observed at the expanding and mature time-point.

Support for the sink to source transition being important for the overall CO_2 response comes from patterns across the entire transcriptional data set. Sixty percent of all the genes encoding enzymes involved in photosynthesis, major and minor carbohydrate metabolism, glycolysis, and the TCA cycle were part of clusters 7 through 10 suggesting that later stages in development where expression peaks at expansion (clusters 9 and 10) or continues to increase over development (clusters 7 and 8) are most important for building the majority of the metabolic machinery important for source tissue (Figure 4.5). These patterns are consistent with those examining a developmental time course of leaf two in Arabidopsis (Skirycz et al. 2010). The broad transcriptional patterns are also in agreement with protein abundance data. In barley leaves, protein abundance for components of the TCA cycle and mitochondrial electron transport chain had similar patterns to clusters 7 through 10 where protein abundance increased until the end of the elongation zone, and then either continued to increase or decreased in photosynthetically mature tissue (Thompson 1998). What is most interesting about these patterns is that 64% of all transcripts significant for a main effect of CO_2 were also part of clusters 7 through 10 (Figure 4.5) and across leaf development the relative difference between ambient and elevated [CO_2] in transcripts coding for the respiratory machinery increased from primordia to mature leaves (Figures 4.6 and 4.7). Therefore as leaves are shifting from importing energy to providing their own energy, the CO_2 effect on the transcriptome is strengthening.

Carbohydrates have been implicated as part of the systemic signal between mature and developing leaves where gene expression changes in expanding tissue were apparent two hours after elevated [CO_2] being applied to mature tissues only (Coupe et al. 2006). The negative feedback on photosynthetic gene expression when excess glucose accumulates in plant cells through a hexokinase-mediated pathway has been demonstrated to be the mechanism behind

photosynthetic acclimation to elevated [CO_2] (Moore et al. 1999). Glucose concentration therefore could be a potential signal for the transcriptional response because the relative differences in glucose concentration between ambient and elevated [CO_2] increased from 23 DAG (+36%) to 30 DAG (+87%; Figure 4.2). Nevertheless, the transcriptional reprogramming of the respiratory machinery in elevated [CO_2] is occurring during the sink-source transition and warrants a more detailed examination of transcript abundance and quantification of how much of those additional transcripts are actively being translated into protein. As in Chapter 3, succinate dehydrogenase 1 (SDH1-1) and aconitate hydratase 2 (ACO2) were significantly greater in elevated [CO_2] and were shown by Lee et al. (2012) to have significantly high correlations ($r > 80$) with protein abundance.

The stimulation in photosynthesis, carbohydrates, respiration, and biomass in elevated [CO_2] in the mature tissue at 30 DAG are consistent with a recent study conducted on Arabidopsis at 35 DAG (Chapter 3). The subtlety of the transcriptional response to elevated [CO_2] across the data set and the transcriptional reprogramming of the respiratory machinery in elevated [CO_2] mirrors previous data sets collected in Arabidopsis (Chapter 3), soybean (Ainsworth et al. 2006, Leakey et al. 2009a) and rice (Fukayama et al. 2011). Taking a broad view of the transcriptional data set across development reveals that while significant differences in any one transcript may exist between ambient and elevated [CO_2] the differences in transcript abundance between developmental time points are generally greater (Figure 4.4). Within any individual time point, significant differences between ambient and elevated [CO_2] were generally not very large indicating that the treatment was much closer to steady state compared to stronger stress or deficiency type experiments (Schieble et al. 2004; Usadel et al. 2008). Although there are significant differences between individual transcripts across development between ambient and elevated [CO_2] there do not appear to be drastic alterations in the leaf developmental program induced by elevated [CO_2].

It is unlikely that there is a single global regulator of the transcriptional response to elevated [CO_2] across all of these plant functional groups, there are interesting patterns that emerge when examining transcription factors that have been previously shown to be involved in key developmental processes in Arabidopsis and are part of clusters 7 through 10 in this experiment (Figure 4.5). A few notable transcription factors that have significantly greater

transcript abundance in elevated [CO_2] across leaf development are WRKY33 (At2g38470; cluster 9), TEMPRANILLO 1 (TEM1; At1g25560; cluster 8), RAV1 (At1g13260; Cluster 7) and WRKY70 (At3g56400; Cluster 9). WRKY 33 was shown to positively regulate aldose-1 epimerase (At4g25900), pyrophosphate-dependent phosphofructo-1-kinase (PFK3; At4g26270) and UDP-glucosyl tranferase (At2g30140) through binding to W-box and/or W-box-like motifs in their promoter regions of these carbon metabolism genes (Jiang and Deyholos 2009) that all were significantly up-regulated in elevated [CO_2]. Thus this transcription factor could be a potential upstream regulator of other CO_2 responsive metabolic genes. TEM1 acts developmentally in both leaves and the shoot apical meristem to delay flowering through the photoperiod pathway (Osnato et al. 2012) by acting antagonistically to CONSTANS by directly binding to the Flowering Time promoter and repressing expression (Castillejo and Pelaz 2008). This gene deserves further investigation as flowering time in Arabidopsis, and many other plants, are altered when grown in elevated [CO_2] (reviewed in Ward and Springer 2007). RAV1 positively and WRKY70 negatively regulate leaf senescence (Woo et al. 2010; Besseau and Palva 2012). Elevated [CO_2] has been shown to delay canopy senescence in soybean and poplar (Miglietta et al. 1993; Dermody et al. 2006; and Taylor et al. 2008). Alterations in the timing of senescence due to elevated [CO_2] have implications for future agricultural and forest productivity. Although the mechanisms by which RAV1 and WRKY70 regulate leaf senescence have only recently started to be elucidated, they may play important roles in the timing of senescence when plants are grown in elevated [CO_2]. Although there is not currently hard molecular evidence that linking these transcription factors for regulating metabolic and major developmental events in elevated [CO_2], they all warrant further investigation through a reverse genetics approach.

Conclusions

In this study we have identified that the physiological increase in dark respiration in the elevated [CO_2] treatment occurs as leaves transition from rapidly expanding sink tissues to mature source tissues in Arabidopsis leaves. The transcriptional reprogramming of the respiratory machinery in response to elevated [CO_2] became more apparent as leaves matured and sugars accumulated in the elevated [CO_2] treatment. This lends further support to the idea that carbohydrate concentration as one of the main signaling mechanisms that regulates gene

expression in elevated [CO_2]. The transcriptional reprogramming of respiration occurred prior to there being a significant difference between ambient and elevated [CO_2] for the leaf level respiration rates suggesting that mitochondrial proteins are actively being made prior to a physiological response. Although there were fewer genes that were significant for the main effect of CO_2 in the primordia than the other two time-points, it is possible that these differentially regulated genes early in leaf development are responsible for some of the downstream changes in gene observed later in leaf development and may act as "master regulators" for the metabolic response. We do not think that there are major fundamental shifts in the leaf developmental program in elevated [CO_2] because it is a relatively mild treatment. It may be that gradual shifts in metabolic optimization scaled across three or more weeks is enough to increase biomass by 30%. Finally, we have identified differentially expressed transcription factors involved in regulating metabolism, which should be targets for follow-up studies.

Methods

Plant Growth Conditions

Arabidopsis thaliana (Col) seeds were soaked in DI water for 15 minutes and planted directly on sterilized LC1 Sunshine Mix (Sun Gro Horticulture, Canada) in 216 cm^3 pots. Planted pots were cold treated at 4^o C for 48 hours prior to being moved into growth chambers. Plants were grown in two identical Conviron (PGR14, Winnepeg, Canada) growth chambers that provided 10/14 hour day/night cycle at 21 °C/18 °C, 70% RH, and 300 μmol m^{-2}/s^{-1} of photosynthetically active radiation. Each individual pot was covered with an upside-down petri dish to raise local relative humidity to ensure uniform germination. Trays of 32 pots were rotated within chambers and between trays every other day to reduce variance in light levels within chambers. Trays and [CO_2] treatment were rotated between chambers every five days to reduce any chamber bias. Pots were watered weekly by adding 1 L of 40% Long Ashton solution containing 6 mM NH_4NO_3 to each tray of pots. To ensure no bias in seedling germination, establishment, and early rosette development, the [CO_2] treatment did not start until 7 days after germination (DAG, growth stage 1.04, Boyes et al. 2001) where plants in both CO_2 treatments reached growth stage 1.10, 17 DAG. Preliminary time-lapse photography data allowed us to determine that leaf 10 would be the youngest mature leaf 30 DAG while still following similar protocols to Coupe et al. (2006). [CO_2] concentration was maintained at ambient (370 ppm) or elevated (750 ppm) using a custom retrofitted chamber CO_2 scrubbing and delivery system. Briefly, [CO_2] in each chamber was sampled continuously every second using an infrared gas analyzer. Ambient [CO_2] was maintained at 370 ppm by routing the growth chamber exhaust and intake through a sealed box containing soda lime (CarboLime, Allied Healthcare) and then adding pure [CO_2] back into the line to get a constant 370 ppm. Elevated CO_2 was maintained at 750 ppm by adding pure [CO_2] to the chamber air intake line using the same delivery system as the ambient chamber.

Physiology, Biochemistry, Specific Leaf Area, Biomass

Leaf dark respiration rates were measured at subjective midnight using a custom designed closed gas exchange system described in detail in Markelz et al. (Chapter 3). Respiration rates were determined on leaf 10 at 18 ^{o}C when leaves were rapidly expanding 23 and 24 DAG and as leaves transitioned into maturity 29, 30, and 31 DAG (n=10-12). After respiration measurements,

leaves were excised, photographed for leaf area and oven dried at 70°C for calculation of Specific Leaf Area (SLA). Whole aboveground tissue was harvested and oven dried for determination of dry biomass. *In-situ* midday rates of photosynthetic [CO_2] assimilation were measured 30 DAG at growth CO_2 concentration on leaf 10 using an open-path LI-6400 portable infrared gas analyzer (n=8; LICOR, NE, USA). Leaf disks (n=8) were collected from leaf 10 at 23 DAG (0.264 cm^2) and 30 DAG (1.2 cm^2), were wrapped in aluminum foil, immediately frozen in liquid N, and stored at -80 °C until carbohydrates and protein were extracted and analyzed as described in Ainsworth et al. (2007).

Gene Expression

Tissue for leaf 10 RNA was harvested at subjective midnight on 16, 23, and 30 DAG. For the leaf primordia time point (16 DAG), whole rosettes were harvested, flash frozen in liquid N, placed in 50 mL conical tubes and stored at -80 °C. RNA *later*-ICE (Invitrogen, NY, USA) stabilizing solution was chilled on dry ice before being added in excess to the conical tubes containing the tissue. The tissue was then allowed to be penetrated by the solution overnight at -20 °C following manufactures protocols. The following day, leaf primordial tissue was dissected using precision forceps under a dissection scope. Twenty individual plants were dissected for each replicate and the tissue was stored in 1.5 mL tubes at -80 °C. Tissue was ground in liquid N chilled 1.5 mL tubes using a chilled plastic pestle aided by acid sterilized fine sand in the Spectra Total Plant RNA Isolation Kit (Sigma, MO USA) extraction buffer. At 23 and 30 DAG, leaf 10 was excised at the base of the blade from eight individual plants per replicate, immediately flash frozen in liquid N and stored at -80 °C until RNA was isolated using the Spectra Kit. Prior to cRNA labeling, total RNA quality was checked for all samples by gel electrophoresis, which confirmed intact ribosomal bands without smearing. The cRNA labeling, the subsequent steps leading up to hybridization and the scanning of the Genechip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA) were performed following manufacturer's protocols at the University of Illinois Keck Center for Functional Genomics (www.biotech.uiuc.edu/centers/Keck).

Statistics

All physiological and biochemical data were analyzed using an ANOVA (PROC GLM, SAS 9.1; SAS Institute, Inc., Cary, NC, USA) where [CO_2] and developmental stage were

considered fixed effects and a p-value <0.05 was used as a significance threshold. Following the detailed protocols of Leakey et al. (2009) and (Chapter 3) for microarray analysis, the transcriptional data set was analyzed using an ANOVA with a 0.05 FDR multi-testing correction (JMP Genomics 5.1; SAS, Cary, NC, USA) where [CO_2] and developmental stage were treated as fixed effects. Individual transcripts were not tested if they were not called present in at least three replicate chips for each [CO_2] by development treatment combination. Genes that were significant for the main effects of [CO_2] and developmental stage or the [CO_2] by developmental stage interaction were visualized on metabolic pathways using MAPMAN functional gene categories (Thimm et al. 2004, Usadel et al. 2005). Hierarchical clustering was performed on all 12,750 transcripts using percentage changes of median normalized intensity values between each developmental stage within each [CO_2] treatment in JMP Genomics. The Ward method was used for clustering with the "Scale Rows" option, which fixed the variance to 1 on a transcript-by-transcript basis so that all transcripts could be viewed on a single heat-map.

Tables and Figures

Table 4.1 The number of transcripts responding significantly ($p<0.05$) to each of the main effects and/or interactions in the ANOVA model of the 12,570 that were represented in at least 3 biological replicate microarray chips.

Factor in ANOVA model	Number of Significant Transcripts
Developmental Time Point	11,337
CO_2 Concentration	2,130
CO_2 by Development Interaction	1,696

Table 4.2 Numeric summary clustering diagram in Figure 4.4 with the breakdown of number of transcripts across the data set that fall into each cluster, a breakdown of transcripts significant for the main effect of CO_2 and the interaction between CO_2 and Development stage. Four functional MAPMAN categories of genes are also displayed. Abbreviations: Tri-carboxylic Acid Cycle (TCA), Mitochondrial Electron Transport Chain (Mito ETC).

Cluster Number	Total Per Cluster	Significant CO_2	Significant C by D	Glycolysis	TCA Cycle	Mito ETC	Photo-synthesis
1	567	62	79	1	2	6	0
2	725	136	117	3	3	12	1
3	1162	224	196	5	6	12	4
4	318	41	61	4	1	6	0
5	1860	195	399	9	7	13	5
6	1400	146	42	0	2	11	2
7	909	236	177	0	4	6	1
8	1958	383	194	9	5	3	16
9	1610	298	132	10	15	9	124
10	2061	416	297	8	14	18	31
TOTAL	12570	2137	1694	49	59	96	154

Table 4.3 List of transcripts that were significant for the main effect ($p<0.05$) of elevated [CO_2] that are displayed in Figures 4.6 and 4.7. AT locus IDs, functional description, and percentage change in elevated [CO_2] versus ambient [CO_2] at primordia (P), expanding (Ex), or mature (M) time-points.

Figure Number	probe_ID	At_number	Function	CO2_P	CO2_Ex	CO2_M
4.5	247338_at	(At5g63680)	glycolysis.pyruvate kinase (PK)	-1.4947857	24.256042	39.10719
4.5	248283_at	(At5g52920)	glycolysis.pyruvate kinase (PK)	8.406781	-24.431023	-20.913738
4.5	249163_at	(At5g42740)	glycolysis.glucose-6-phosphate isomerase	4.870071	-22.040972	-6.1726127
4.5	253966_at	(At4g26520)	glycolysis.aldolase	3.5824797	-5.6655912	-18.246408
4.5	253987_at	(At4g26270)	glycolysis.phosphofructokinase (PFK)	15.022014	49.30785	29.82651
4.5	254141_at	(At4g24620)	glycolysis.glucose-6-phosphate isomerase	5.118894	-26.083672	-23.752392
4.5	255033_at	(At4g09520)	glycolysis.phosphoglycerate mutase	-14.836331	4.274038	-19.57756
4.5	256836_at	(At3g22960)	glycolysis.pyruvate kinase (PK)	4.5157237	-24.054995	-18.440039
4.5	258588_s_at	(At3g04120)	glycolysis.glyceraldehyde 3-phosphate DH (GAP-DH)	-0.25350863	12.309581	37.58536
4.5	259969_at	(At1g76550)	glycolysis.pyrophosphate-fructose-6-P phosphotransferase	-6.9710274	-8.743324	-9.555166
4.5	260590_at	(At1g53310)	glycolysis.phospho-enol-pyruvate carboxylase (PEPC)	-0.5232003	2.6171598	27.109247
4.5	262944_at	(At1g79550)	glycolysis.3-phosphoglycerate kinase (PGK)	2.6229925	2.5708654	21.432198
4.5	266266_at	(At2g29560)	glycolysis.enolase	-5.8783836	-13.162746	-8.136669
4.6	246035_at	(At5g08300)	TCA succinyl-CoA ligase	-2.126242	4.6750784	20.16477
4.6	246396_at	(At1g58180)	TCA carbonic anhydrases	-0.42261738	32.568592	22.900007
4.6	247060_at	(At5g66760)	TCA succinate dehydrogenase	2.1033356	3.2209632	26.038282
4.6	248461_s_at	(At2g47510, at5g50950)	TCA fumarase	1.5862399	-2.1759567	-14.655978
4.6	248608_at	(At5g49460)	TCA atp-citrate lyase	1.0376507	-21.970585	-22.332872
4.6	250339_at	(At5g11670)	TCA organic acid transformaitons.malic	3.3899531	30.285181	46.178284
4.6	250929_at	(At5g03290)	TCA organic acid transformaitons.IDH	1.3801357	10.200976	15.1482115
4.6	253135_at	(At4g35830)	TCA aconitase	6.2634897	5.7671785	11.769224
4.6	253196_at	(At4g35260)	TCA IDH	9.327929	7.547948	20.409063
4.6	253300_at	(At4g33580)	TCA carbonic anhydrases	-7.8172956	-11.660719	-19.209307
4.6	253954_at	(At4g26970)	TCA aconitase	8.553632	22.972778	26.294516
4.6	256160_at	(At1g30120)	TCA pyruvate DH.E1	-2.9180496	-9.970482	-17.607723
4.6	257895_at	(At3g16950)	TCA pyruvate DH.E3	0.8667725	-26.7643	-24.202614
4.6	258439_at	(At3g17240)	TCA pyruvate DH.E3	5.5466657	5.528816	7.9034505
4.6	259161_at	(At3g01500)	TCA carbonic anhydrases	-8.190904	-10.173377	-13.404858
4.6	261165_at	(At1g34430)	TCA pyruvate DH.E2	7.5662136	-25.413523	-28.742735

			Table 4.3 (cont.)			
4.6	261583_at	(At1g01090)	TCA pyruvate DH.E1	-6.5607886	-14.677627	-16.229918
4.6	261833_at	(At1g10670)	TCA atp-citrate lyase	-5.5433025	-2.748123	-4.4420376
4.6	263583_at	(At2g17130)	TCA IDH	3.9514585	19.202707	38.2857
4.6	263663_at	(At1g04410)	TCA cyt MDH	2.2761672	8.620996	7.4297075
4.6	267368_at	(At2g44350)	TCA CS	5.8686175	4.816875	9.411243
4.6	247746_at	(At5g58970)	ETC.uncoupling protein	-6.497555	-4.0991173	-6.8844514
4.6	252864_at	(At4g39740)	ETC.cytochrome c oxidase	10.032904	17.308699	13.215384
4.6	253810_at	(At4g28220)	ETC.NADH-DH.type II.external	4.0217347	-7.9783645	-17.426487
4.6	255011_at	(At4g10040)	ETC.cytochrome c	2.7967746	11.551893	19.758827
4.6	255259_at	(At4g05020)	ETC.NADH-DH.type II.external	-14.9083185	87.31157	94.937416
4.6	255442_at	(At4g02580)	ETC.NADH-DH	-8.128773	-18.95596	0.8753025
4.6	257339_s_at	(Atmg00040, at2g07671)	ETC.F1-ATPase	-13.25034	33.349277	52.676285
4.6	258164_at	(At3g17910)	ETC.cytochrome c oxidase	-2.274972	20.9097	24.302042
4.6	259594_at	(At1g28140)	ETC.cytochrome c oxidase	-1.1273777	-6.281095	-9.356404
4.6	260418_s_at	(At1g69750, at1g66590)	ETC.cytochrome c oxidase	-6.639836	21.182468	37.9818
4.6	266012_s_at	(At2g07699)	ETC.F1-ATPase	13.725044	15.313386	36.034256

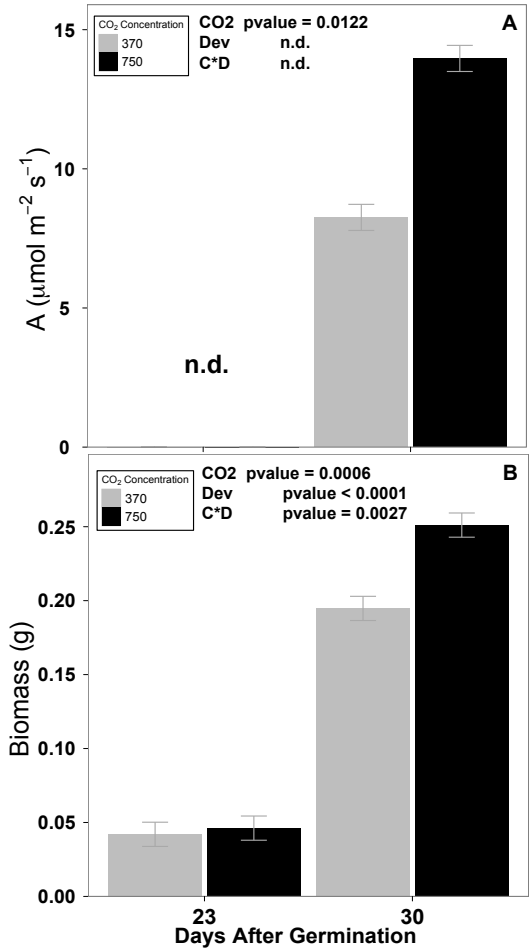

Figure 4.1 Midday in-situ CO_2 assimilation (A) at growth [CO_2] (Panel A) and Total above ground dry biomass (B). Mean values (+/- standard errors) of physiological parameters of leaf 10 grown in ambient (370 ppm) or elevated (750 ppm) [CO_2] at 23 (Expanding) or 30 (Mature) days after germination. Photosynthesis was not determined (n.d.) in the expanding leaves at 23 DAG due to technical limitations. Also plotted are the p-values from the statistical model each of the parameters.

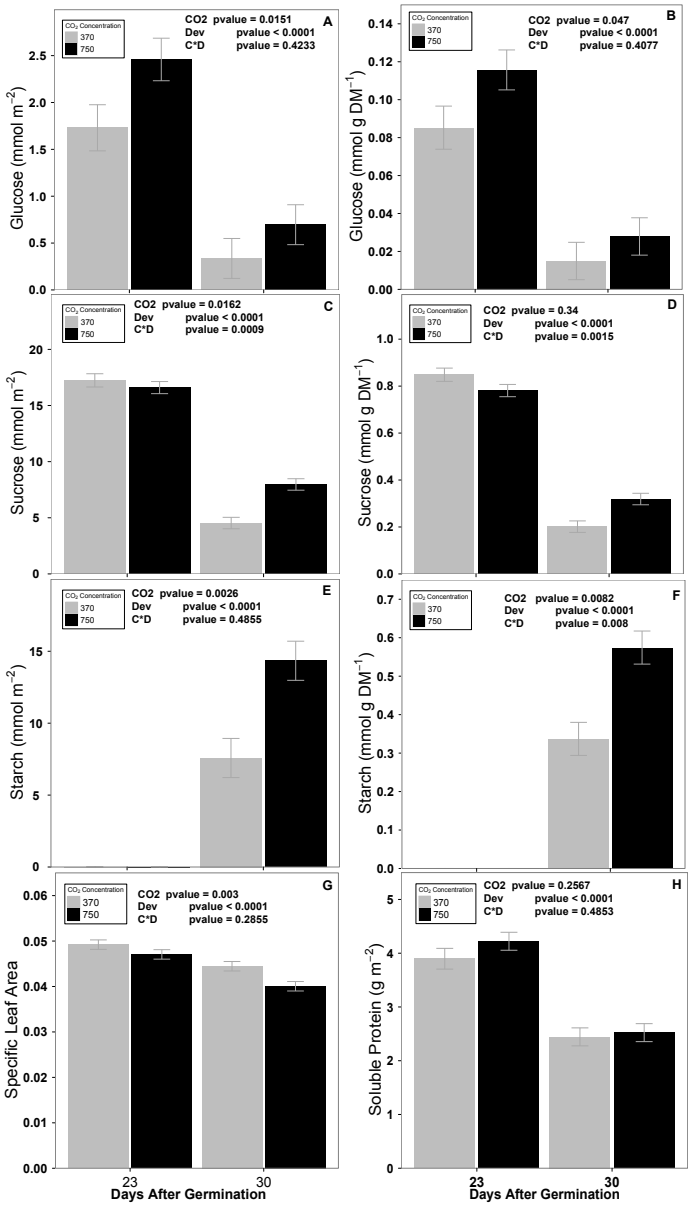

Figure 4.2 Midnight glucose content (A), midnight glucose concentration (B), midnight sucrose content (C), midnight sucrose concentration (D), midnight starch content (E), midnight starch concentration (F), Specific leaf area (G), and leaf soluble protein content (H). Mean values (+/- standard errors) of physiological parameters of leaf 10 grown in ambient (370 ppm) or elevated (750 ppm) [CO_2] at 23 (Expanding) or 30 (Mature) days after germination. Also plotted are the p-values from the statistical model from each of the parameters.

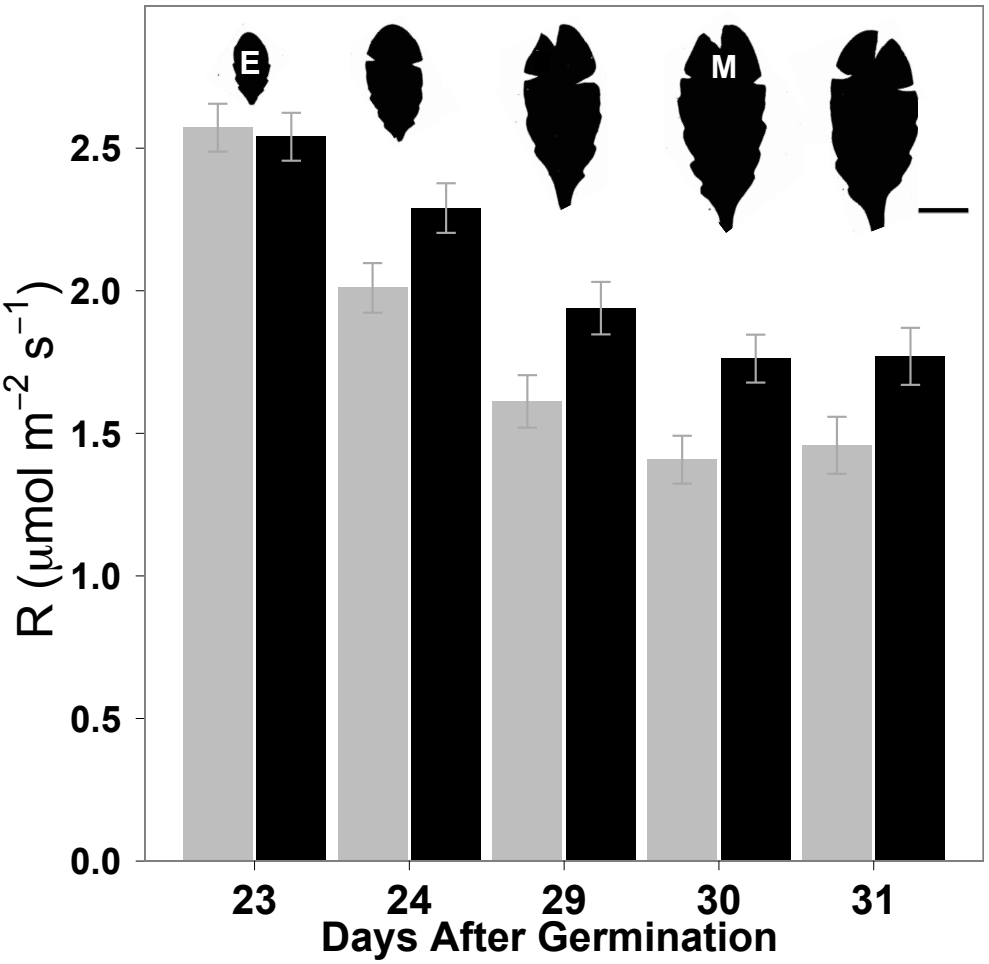

Figure 4.3 Mean values (+/- standard errors) of midnight dark respiration rates (R) of leaf 10 grown in ambient (grey bars; 370 ppm) or elevated (black bars; 750 ppm) [CO_2] at 23 (Expanding; E), 24, 29, 30 (Mature; M), 31 days after germination (DAG). The p-values from the statistical model each of the parameters are: CO_2 $p < 0.0001$; Development $p < 0.0001$; CO_2 by Development $p = 0.1486$. To show relative leaf sizes at each developmental stage, representative leaf areas are shown above each time point. As leaves aged it was necessary to cut the leaf blades slightly to get them to lie flat for imaging. Scale bar = 1 cm.

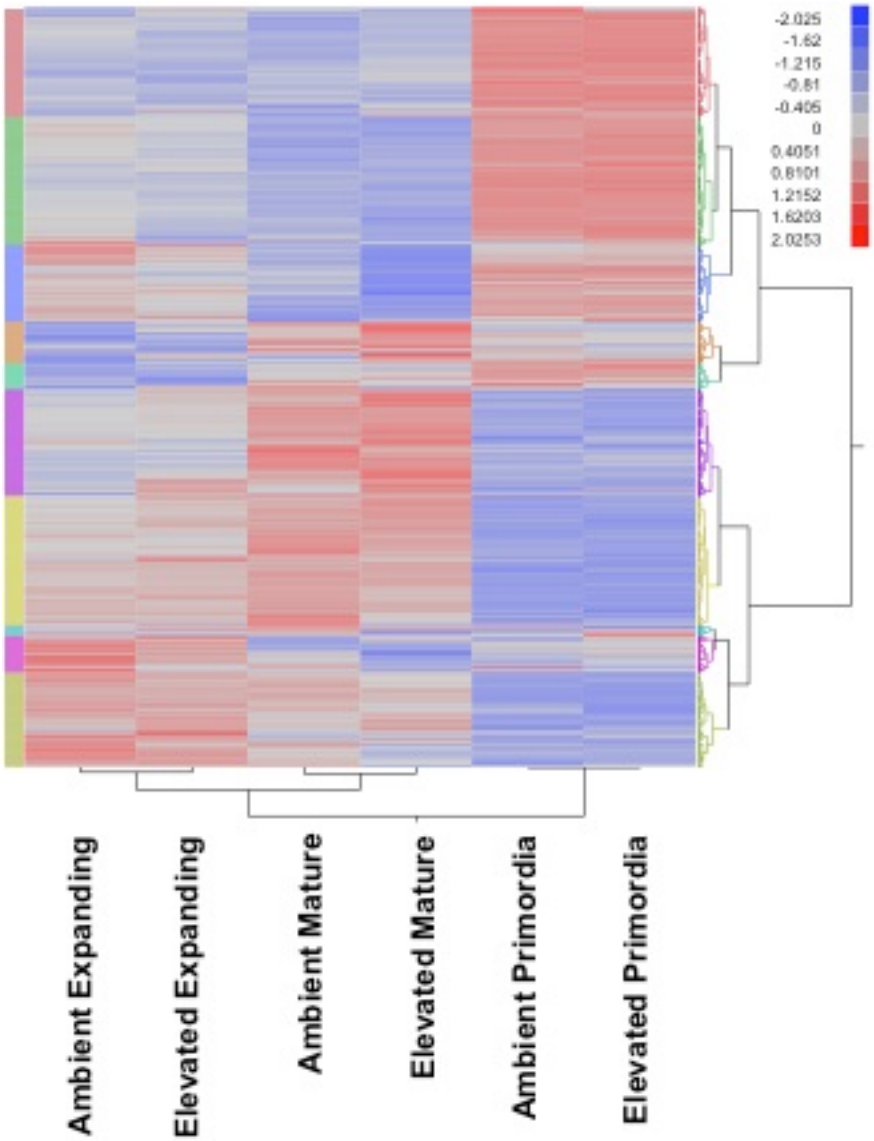

Figure 4.4 Hierarchical clustering of normalized mean fluorescence intensity values (Log_2) of the 12, 570 transcripts of this leaf developmental time course study in ambient and elevated CO_2. Blue indicates lower intensity and red indicates greater intensity compared to the mean intensity values (gray). Tissue was harvested from leaf 10 at midnight on 16 (Primordia), 23 (Expanding), and 30 (Mature) days after germination from the same leaf cohort as the other leaf biochemical and physiological parameters.

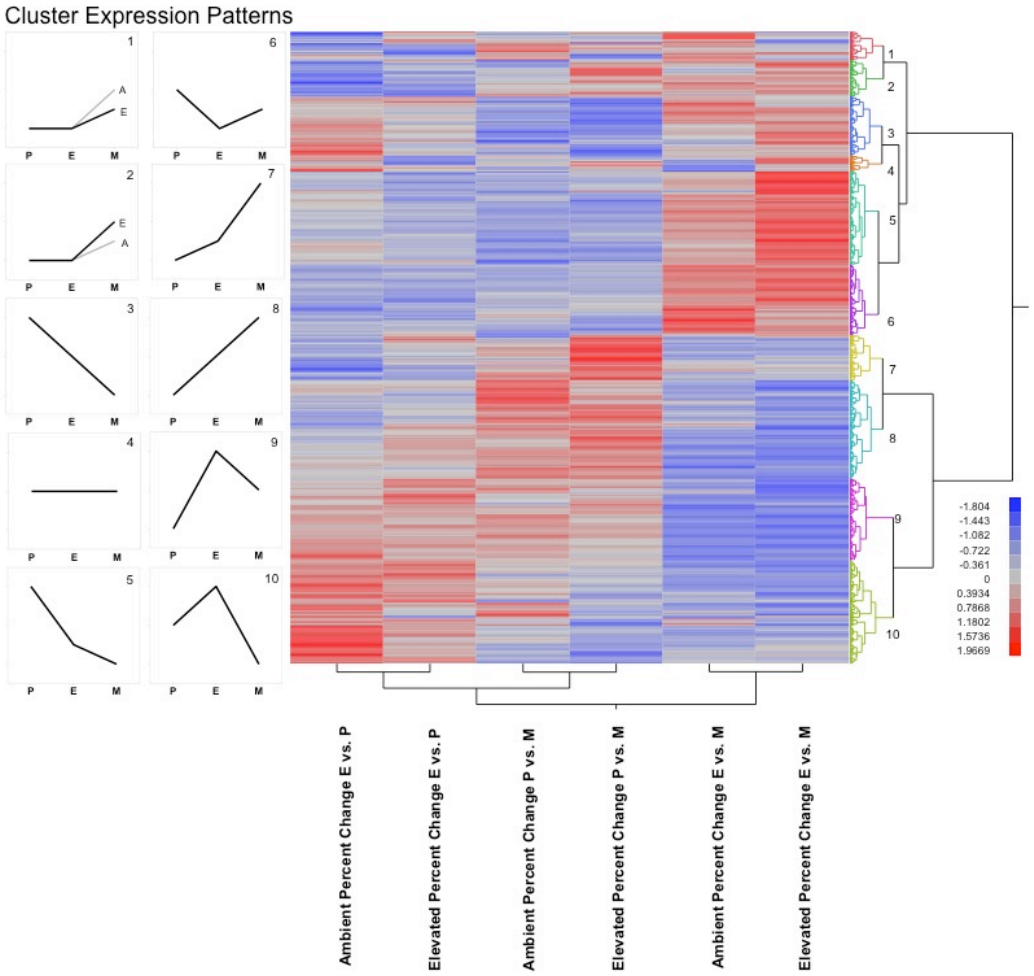

Figure 4.5 Hierarchical clustering of the percentage change between developmental time points within each ambient or elevated [CO_2] treatment. All 12,570 transcripts of this leaf developmental time course study were used for this analysis. Blue indicates lower intensity and red indicates greater intensity compared to the mean intensity values (grey). Tissue was harvested from leaf 10 at midnight on 16 (Primordia; P), 23 (Expanding; E), and 30 (Mature; M) days after germination. Transcripts were grouped into clusters based on the 10 major expression patterns between developmental time points illustrated graphically on the left hand portion of the figure. The y-axis of these clusters is only used to illustrate relative expression patterns between time points. In clusters 1 and 2 there were small differences between ambient (grey) and elevated [CO_2] (black) at the mature time point, but for the remaining clusters black represents ambient and elevated [CO_2].

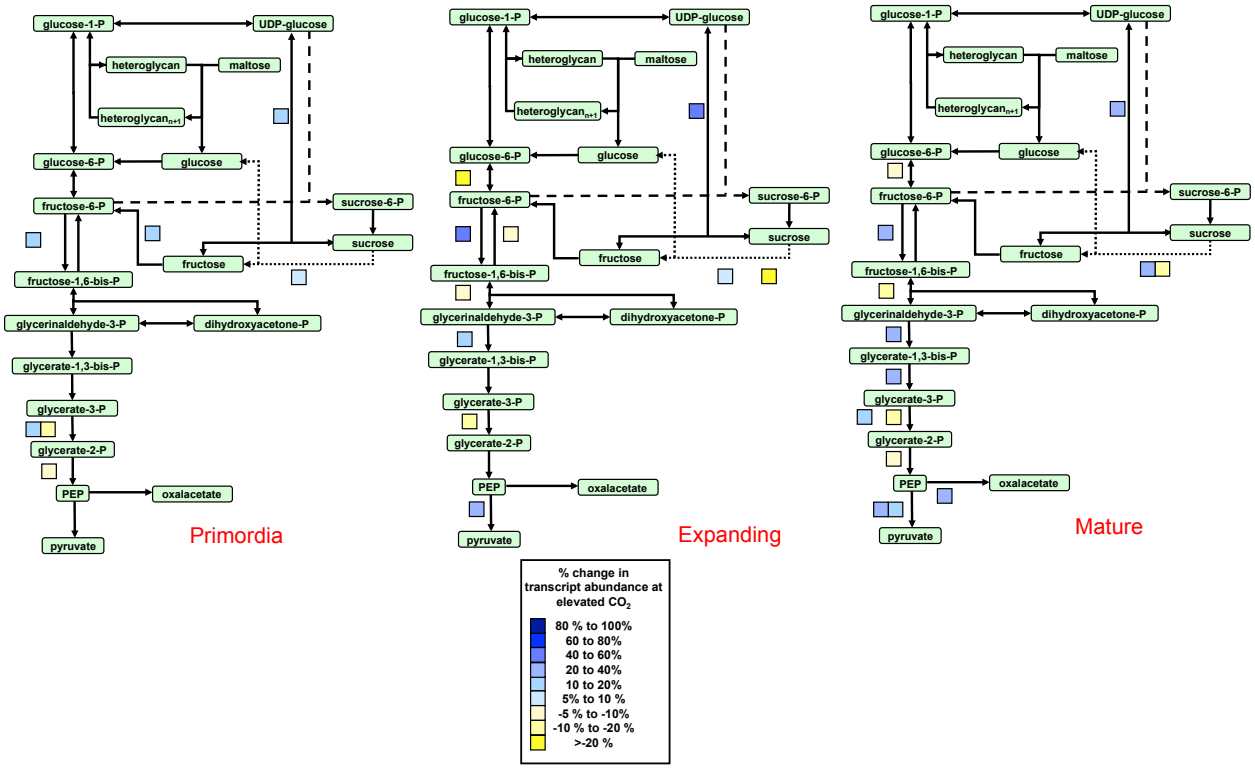

Figure 4.6 A graphical representation of genes encoding components of sugar transformations reactions and glycolysis. Tissue was harvested from leaf 10 at midnight on 16 (Primordia), 23 (Expanding), and 30 (Mature) days after germination. Each blue and yellow box represents the mean value of a unique transcript that responded significantly ($P < 0.05$) in elevated [CO_2]. Blue is greater transcript abundance for that gene at elevated [CO_2] compared to ambient [CO_2] and yellow is less transcript abundance for those genes at elevated [CO_2]. Details about individual transcripts can be found in Table 4.3.

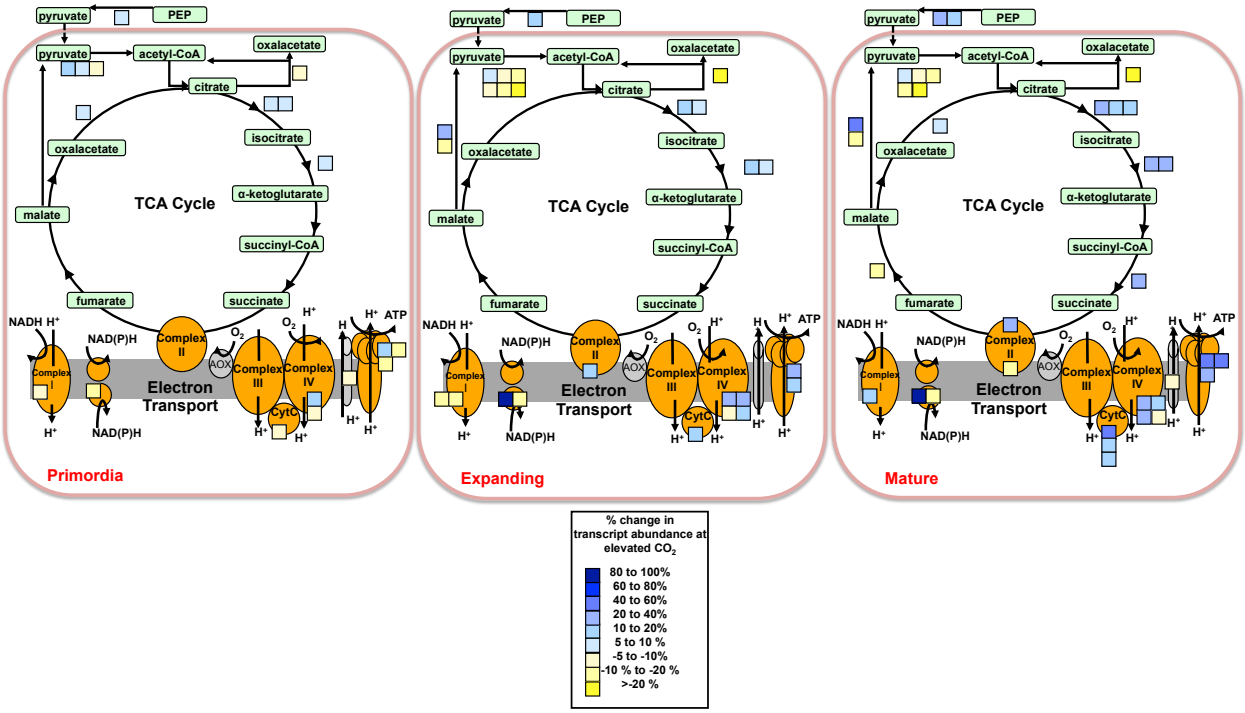

Figure 4.7 A graphical representation of genes encoding components of the TCA cycle and the mitochondrial electron transport chain. Tissue was harvested from leaf 10 at midnight on 16 (Primordia), 23 (Expanding), and 30 (Mature) days after germination. Each blue and yellow box represents the mean value of a unique transcript that responded significantly ($P < 0.05$) to elevated [CO_2]. Blue is greater transcript abundance for that gene at elevated [CO_2] compared to ambient [CO_2] and yellow is less transcript abundance for those genes at elevated [CO_2]. Details about individual transcripts can be found in Table 4.3.

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CHAPTER V: CONCLUDING REMARKS

A significant challenge to understanding global climate change impacts on plants and ecosystems is scaling between a mechanistic understanding at the molecular level and putting that mechanism in a broader ecological context. On the broad end of the scale, environmental change factors such as atmospheric [CO_2], soil water availability, and nitrogen availability all interact to affect plant productivity (Gerten et al. 2004; Ainsworth and Long 2005; Ghannoum 2009; Leakey et al. 2009; Soussana et al. 2010). On the narrow end of the scale, these environmental factors are interacting directly with individual plant cells through molecular and biochemical mechanisms that scale up to the whole plant (Leakey et al. 2009). A way to bridge these large gaps in biological organization is by understanding the impacts that these climate change factors have on plant physiology, but to do so using ecologically relevant treatments. While the questions addressed in this dissertation's data chapters have ranged in focus from C_4 plants in a field setting to C_3 plants in a highly controlled environment chamber, the overarching theme has been on advancing mechanistic understanding of the effects of elevated [CO_2] on the leaf level physiology of these plants.

Broadly examining the literature on the effects of elevated [CO_2] on leaf level physiology reveals that there are responses that are largely conserved across a broad range of species tested. Two important leaf level responses to elevated [CO_2] that are directly related to my dissertation research are: (1) the ubiquitous reduction in stomatal conductance in both C_3 and C_4 species; (2) the direct stimulation of C_3 photosynthesis (reviewed in Ainsworth and Long 2005). Observing these same leaf level responses in the experiments conducted in the data chapters provided anchor points for the understanding and interpretation of the experimental results. For example, in Chapter 2 the reduction in stomatal conductance in elevated [CO_2] reduced the amount of water passing through the stomates that gradually over time increased the amount of soil moisture available to the elevated [CO_2] plants compared to the ambient [CO_2] plants. Or, in Chapters 3 and 4 the stimulation of photosynthesis in elevated [CO_2] led to greater carbohydrate availability that is likely stimulating the observed respiratory response and downstream increases in biomass. Knowledge of these anchor points allowed scaling up to the whole plant level (Chapter 2) and scale down to the molecular level (Chapters 3 and 4) to answer open questions about the interaction of elevated [CO_2] with N availability, drought, and development.

Understanding the main effects of individual climate change variables on plants was used as an anchor point that allowed exploration of elevated [CO_2] with other interacting factors in each data chapter. Recent modeling, long-term ecological studies, and synthesis papers conclude that significant interactions of elevated [CO_2] with water and nitrogen availability are key drivers to plant and ecosystem responses to climate change (Reich et al. 2006; Lou et al. 2008; Leakey et al. 2009; McCarthy et al. 2010; Norby et al. 2010; Norby and Zak 2011; Leakey et al. 2012). For example, the finding that N availability in longer-term [CO_2] enrichment studies on ecosystems limits the stimulatory effects of elevated [CO_2] on ecosystem productivity (Reich et al. 2006; McCarthy et al. 2010; Norby et al. 2010). These ecosystem-scale studies provided the ecological context and motivation for a more detailed physiological and molecular investigation of the mechanisms underlying the observed responses. At the molecular end of the scale, the maturation of high throughput -omic technologies has allowed for quantification of hundreds/thousands of metabolites, transcripts, and proteins simultaneously. Significant advances in understanding how carbon metabolism responds to environmental variables and to developmental gradients have come from using –omic techniques (Schieble et al. 2004; Ainsworth et al. 2006; Leakey et al. 2009a; Gibon et al. 2009; Tschoep et al. 2009; Skirycz et al. 2010; Witt et al. 2012; Farre and Weise 2012). For example, the strong role that the circadian clock has on regulating gene expression of one-third of Arabidopsis genes (Covington et al. 2008) and the circadian clock's role in modulating starch accumulation and breakdown to coordinate plant growth with carbohydrate supply on a diel cycle (Smith and Stitt 2007). Yet molecular studies investigating interactions between carbon metabolism and nitrogen availability or drought typically utilize unrealistically acute treatments (Shieble et al. 2004; Usadel et al. 2008; Gibon et al. 2009; Skirycz et al. 2010; Witt et al. 2012) thus reducing the ecological relevance of the findings. Therefore this thesis attempted to incorporate these molecular techniques into the design and interpretation of eco-physiologically based experiments and vis-a-versa to integrate these levels of biological organization.

Chapter 2 addressed the uncertainty about how the effect of elevated [CO_2] on C_4 maize photosynthesis yield depends on N availability and drought stress. A detailed record of soil moisture over the course of the growing season allowed links between leaf level changes in stomatal conductance in elevated [CO_2] with greater soil moisture belowground. These soil

moisture savings partially delayed the onset of a natural drought for the plants growing in elevated [CO_2] leading to less of a reduction in the photosynthetic capacity. Elevated [CO_2] also helped ameliorate drought stress by increasing the internal [CO_2] inside the leaves for a given stomatal conductance. The effects of limiting N and elevated [CO_2] were additive, so amelioration of stress by elevated [CO_2] did not differ in magnitude between high N and limiting N supply. The results of this study supported hypotheses put forward by Leakey 2009, but for which there was no previous experimental evidence. In addition, this study extended the inference space of the effects of elevated [CO_2] on C_4 photosynthesis in the field to include a limiting nitrogen availability treatment. The main conclusions that can be drawn is that water relations are a main driver of the C_4 photosynthetic response to elevated [CO_2] across a wide range of N availabilities and therefore much of the US maize growing region. While these general conclusions about the leaf level physiology are supported across multiple field seasons there has not been an observed enhancement in yield in elevated [CO_2] at the SoyFACE facility (Leakey et al. 2004, 2006; Chapter 2). The significance of these findings from open-air field conditions is very important as it contrasts with a significant stimulation of C_4 photosynthesis to elevated [CO_2] observed across multiple species grown in 3-5 liter pots (Ziska and Bunce 1997; Ziska et al. 1999; Maroco et al. 1999). Important economic C_4 species like maize, millet, and sorghum can have rooting depths of up to 2 meters that greatly exceeds the rooting volume of most pots (Allen et al. 1998; Carcova et al. 2000) thus bringing into question if these mature plants were pot bond (Masle et al. 1990) and/or sufficient water was available even through daily watering (reviewed in Leakey 2009). Nevertheless, the stomatal and non-stomatal factors contributing to the amelioration of drought stress identified in Chapter 2 are likely physiological mechanisms underpinning the stimulation in aboveground biomass in C_4 grasses growing in elevated CO_2 during dry years (Wand et al. 2001; Conley et al. 2011). Future research should examine the [CO_2] by drought interactions on yield when there is a much more sustained natural drought (e.g. 2012).

Chapter 3 addressed the question about the role of N availability in modulating the response of C_3 leaf respiration to elevated [CO_2]. In general, the effect of elevated [CO_2] on plant respiration has been a contentious issue in the literature (Drake et al. 1999, Wang and Curtis 2002, Gifford 2003, Davey et al. 2004, Gonzalez-Meler et al. 2004; Leakey et al. 2009a,b).

Given that a majority of the literature justifications for why leaf respiration should change in elevated [CO_2] revolved around tissue N content, the experiment in chapter 3 was explicitly designed to test this interaction. However, before accomplishing this, consideration of measurement artifacts that were most likely introduced into many data sets because inadequate equipment was used in the past to quantify respiration rates (Jahnke et al. 2001; Davey et al. 2004). Through careful design of custom gas exchange equipment that avoided these artifacts, very small (12%) treatment differences between ambient and elevated [CO_2] in the limiting N treatment could be detected in Chapter 3. These findings, along with the 30% stimulation of respiration in the elevated [CO_2] ample N treatment, point to the conclusion that N availability does modulate the response of leaf level respiration to elevated [CO_2] and may be a contributing factor to the observed variance in responses previously reported. The general transcriptional stimulation of the respiratory pathway in elevated [CO_2] indicates that similar transcriptional regulatory networks are being engaged across both levels of N. This fact, along with the data sets from field-grown soybean (Ainsworth et al. 2006; Leakey et al. 2009a) and rice (Fukayama et al. 2011) suggest that there is a conserved mechanism of transcriptional regulation across a wide range of herbaceous species that helps regulate response to a sustained increase carbon availability. Furthermore, because the greatest transcriptional response to elevated [CO_2] across the data set was in ample N supply during the day versus the night there may be significant interactions with the overall [CO_2] response with the circadian clock. This may be important because of the observation that a majority of leaf proteins are estimated to turnover during the daylight hours (Piques et al. 2009). Therefore future research directions should include investigations of the transcriptional and physiological responses of mutant lines in the central oscillators of the circadian clock.

Much of the research examining genes underlying the physiological responses to climate change factors has focused on fully mature leaves (Taylor et al. 2005; Leakey et al. 2009; Fukayama et al. 2011; Gillespie et al. 2012; Chapter 3). However, if transcriptional reprogramming of key metabolic processes is occurring earlier in leaf development to build physiological capacities, these data sets are limited in the conclusions they can draw. In particular the transcriptional “master switches” of acclimation to elevated [CO_2] are unlikely to have been identified. A very forward thinking conceptual model of effects of elevated [CO_2] on

plant growth and development was put forth over a decade ago by Pritchard et al. (1999) that put meristem function and gene expression as the hub between physiology, growth, and development. This model implied that signaling to the meristem from mature tissues could influence the developmental program. Follow-up studies provided evidence for the existence of a systemic signal transmitted from mature tissues to the meristem that would influence gene expression and developmental programs in response to elevated [CO_2] (Lake et al. 2001, Coupe et al. 2006). While Ainsworth et al. (2006) provided the first evidence for transcriptional reprogramming of the respiratory pathway in elevated [CO_2] occurring prior to leaf maturity, the leaf level respiratory fluxes were not measured. The experiment in Chapter 4 was specifically designed to examine the effects of elevated [CO_2] on respiration across a leaf developmental time sequence. The findings suggest that a sink to source transition is necessary for a stimulatory effect on leaf respiration in elevated [CO_2]. This is further supported by the transcriptional data with a majority of the genes that are significant for a main effect [CO_2] in the respiratory pathway showing a greater difference in expression as the leaf matures and builds photosynthetic machinery. These data imply that the greater respiration rates in mature tissues are in part due to building more mitochondrial proteins earlier in leaf development when respiratory demands are high to produce carbon skeletons and energy to support both growth and maintenance processes (Lambers et al. 2008). Linking the gene expression patterns to potential changes in protein abundance would be an important next step. In looking at the data set more broadly, four transcription factors that have consistently greater expression in elevated [CO_2] in this study and in Chapter 3 that have been shown to regulate metabolic genes or be key contributors to the timing of flowering and leaf senescence were identified. A next important step would be to determine if any of these transcription factors contribute to the differences in metabolism, flowering, or leaf senescence using a reverse genetics approach.

In summary, this dissertation has addressed key knowledge gaps in understanding of the effects of elevated [CO_2] on leaf physiology in C_3 and C_4 plants through investigating interactions with nitrogen availability, drought, and a developmental time course. The conclusions that can be drawn from this research that: (1) the effects of elevated [CO_2] on C_4 maize photosynthesis are limited to times and places of drought stress, regardless of N availability, and are mediated by both stomatal and non-stomatal factors; (2) the stimulation of

C_3 respiration and transcriptional reprogramming of respiration at elevated [CO_2] is attenuated in limiting N, but not eliminated; (3) transcriptional reprogramming of leaf metabolism starts in the primordial and continues until leaf maturity, but stimulated flux through the respiratory pathway is only observed after the transition of the leaf from a carbon sink to a carbon source. These conclusions have consequences for the accurate prediction of future food supply, ecosystem function, and providing a leaf level physiological view of these interactions that could be the basis for adapting crops to cope with global environmental change.

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