THE ROLE OF ENVIRONMENT, SINK CAPACITY, AND CARBON TRANSLOCATION 
IN DETERMINING C₃ PLANT RESPONSES TO ELEVATED [CO₂]

BY

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ABSTRACT

Over the past century, CO₂ concentration ([CO₂]) in the atmosphere has been steadily increasing, leading to global climate change. Elevated [CO₂] increases yield, biomass, and photosynthesis in most C₃ plants, but the degree to which elevated [CO₂] stimulates crop yields can depend upon climatic factors and plant physiological attributes, including sink strength and sugar transport capacity. This thesis uses field, laboratory, and meta-analytic techniques to investigate factors that influence the responsiveness of plants to elevated [CO₂], with the ultimate aim of understanding variation in and improving future crop production.

Photosynthesis is typically stimulated in C₃ crops exposed to elevated [CO₂], while stomatal conductance is typically decreased. Theory predicts that the magnitude of stimulation of photosynthesis at elevated [CO₂] is greater at higher temperatures. In Chapter 2, the degree to which these physiological responses of C₃ crops to elevated [CO₂] would translate to yield responses was tested. Using a global dataset of published yield data from Free Air CO₂ Enrichment (FACE) and Open Top Chamber (OTC) experiments, there was greater yield response to elevated [CO₂] in C₃ crops under dryer conditions, but there was no correlation between yield response to elevated [CO₂] and growing season temperature. Thus, the theoretical response of photosynthesis to elevated [CO₂] and temperature was not observed in seed yield, perhaps due to direct effects of temperature on respiration or reproductive processes.

In Chapter 3, intraspecific variation in soybean (Glycine max) response to elevated [CO₂] and the agronomic traits associated with greater yield responsiveness to elevated [CO₂] were analyzed. Eighteen soybean cultivars, varying in maturity group, year of release date, and agronomic traits, were grown at SoyFACE from 2003 to 2008. There was significant intraspecific variation in yield response to elevated [CO₂], with shorter cultivars and those with high harvest index showing greater response to elevated [CO₂]. Harvest index is an indicator of sink strength, which may be important for CO₂ response, because it can relieve the accumulation of carbohydrates in the photosynthesizing leaves, which at high concentrations in elevated [CO₂] can signal down-regulation of photosynthetic capacity.

In Chapter 4, the hypothesis that different mechanisms of phloem loading can lead to a change in the photosynthetic response to elevated [CO₂] was tested. Plants have evolved different strategies to load phloem with sugars to send to sink tissue. One method, apoplastic loading, uses active sugar transporters to load phloem, while another method, symplastic loading,
uses passive diffusion along a sucrose gradient from leaf mesophyll cells to phloem. The hypothesis was that passive loaders, adapted to high mesophyll sucrose concentrations, would experience less sugar-mediated feedback of photosynthesis at elevated $[\text{CO}_2]$ compared to apoplastic loaders. To test this, *Pisum sativum* (pea) and *Beta vulgaris* (beet; apoplastic phloem loaders) and *Fragaria x ananassa* (strawberry) and *Paeonia lactiflora* (peony; passive phloem loaders) were grown at elevated $[\text{CO}_2]$ in the field in 2013 and 2014, testing their biochemical, photosynthetic, and growth responses. All species responded to elevated $[\text{CO}_2]$ with increased photosynthesis and little down-regulation of capacity. There was a strong stimulation in leaf starch but little increase in leaf soluble sugar content at elevated $[\text{CO}_2]$, suggesting little sugar mediated downregulation of photosynthesis in any species. Thus, phloem loading strategy does not appear to be a strong determinant of plant response to elevated $[\text{CO}_2]$.

In Chapter 5, the impact of phloem loading on response to elevated $[\text{CO}_2]$ was studied further in two transgenic lines of *Arabidopsis thaliana* with altered sucrose transporter expression. In the HvSUT1 genotype, the primary sucrose transporter used for phloem loading in *Arabidopsis* (AtSUC2), was replaced with a barley sucrose transporter (HvSUT1), driven by the native AtSUC2 promoter since *in vitro*, HvSUT1 is more active than AtSUC2. In the AtSUC1 genotype, AtSUC1 was overexpressed in a wild-type background using the viral companion cell-specific promoter CoYMV to increase sucrose transporter expression. Neither transgenic line showed improved growth at ambient or elevated $[\text{CO}_2]$ compared to wild-type. The AtSUC1 genotype had a much greater response to elevated $[\text{CO}_2]$ than the other two genotypes, but only because growth at ambient $[\text{CO}_2]$ was significantly reduced. The reasons for stunted growth at ambient $[\text{CO}_2]$ in AtSUC1 are not clear, but do not appear to be related to phosphate limitation.

This dissertation research provides insight into the physiological mechanisms behind the response of plants to elevated $[\text{CO}_2]$. Across field experiments, water availability significantly alters response to elevated $[\text{CO}_2]$, with drier experiments showing a greater response. Within the soybean germplasm, height and partitioning coefficient both correlate to response to elevated $[\text{CO}_2]$. There did not, however, appear to be a link between phloem loading strategy and response to elevated $[\text{CO}_2]$ and phloem loading capacity had mixed effects on response to elevated $[\text{CO}_2]$. This research will be important for better estimating and maximizing response to elevated $[\text{CO}_2]$. 

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CHAPTER I: GENERAL INTRODUCTION

Since the Industrial Revolution, atmospheric carbon dioxide concentration ([CO₂]) has been increasing and, given current emission trends, is expected to continue to increase (Canadell et al. 2007; Le Quere et al. 2009). This increase in [CO₂] has been caused by land use change, such as deforestation, which has been largely constant since 1960, and consumption of fossil fuels, which has nearly quadrupled since 1960 (Le Quere et al. 2009; Ciais et al. 2013). In 1860, atmospheric [CO₂] was ~280 ppm, but by 2013, it was ~400 ppm, and it is expected to exceed 500 ppm by 2050 (Ciais et al. 2013). This increase in [CO₂] is the major contributing factor to global warming (Ciais et al. 2013), but can also directly stimulate plant photosynthesis, biomass, and crop yield (Kimball et al. 2002; Ainsworth & Long 2005).

Many field experiments on plant responses to elevated [CO₂] have been conducted over the past 30 years (reviewed by Ainsworth & Long 2005; Leakey et al. 2012; Bishop et al. 2014). The two most common methods of exposing plants rooted in the ground to elevated [CO₂] are Open Top Chambers (OTC) and Free Air CO₂ Enrichment (FACE). OTCs generally have plastic, transparent walls surrounding a given area and pump CO₂ into that area to a desired concentration (Leadley & Drake 1993). OTCs can increase temperature, decrease light intensity, and decrease wind velocity, therefore changing the microenvironment, but they provide an approximation of outside conditions and can be highly replicated in the field (Leadley & Drake 1993; Long et al. 2004). FACE technology uses an array of pipes that release CO₂ into the wind under fully open air conditions. Flow rate and direction of exhaust are determined by wind direction, velocity, and measured [CO₂] inside the octagonal or circular plot (Miglietta et al. 2001b). Since there is no enclosure, plots can have the same microenvironment as the outside field and plots can be much larger than open top chambers. Smaller mini FACE plots have been developed as well (Miglietta et al. 2001a; Högy et al. 2009).

Open top chamber and FACE experiments have been performed in many locations across the globe (Fig. 1.1) and at a range of temperatures and precipitation levels (Fig. 1.2). Although there are prominent gaps in tropical and arctic regions (Leakey et al. 2012), this dataset can be used to determine how the response to elevated [CO₂] changes with environmental conditions, better informing models and future experiments. Theoretically, the response of C₃ plants to elevated [CO₂] could be greater at high temperatures since the affinity of the Rubisco protein for CO₂ over O₂ declines at higher temperatures (Long 1991). When [CO₂] increases at the site of
Rubisco, it can increase the velocity of carboxylation and decrease oxygenation, therefore decreasing photorespiration (Drake et al. 1997; Leakey et al. 2009a). Thus, the modeled temperature optimum of photosynthetic C assimilation is greater at elevated [CO₂] (Long 1991). However, whether this theoretical photosynthetic response to elevated [CO₂] and temperature extends to crop seed yield production is unknown. When the interaction between temperature and elevated [CO₂] was tested in field experiments, the results were mixed. In rice, the response of yield to elevated [CO₂] was negatively correlated with growing season temperature (Hasegawa et al. 2013). When a factorial experiment of elevated temperature and elevated [CO₂] plots in soybean was performed, there was a greater stimulation in biomass and yield in the elevated temperature plots in only one of the two years studied, and it was the cooler of the two years that showed greater yield at elevated [CO₂] under higher temperatures (Ruiz-Vera et al. 2013). These results suggest that the temperature optimum for reproductive development and seed yield is lower than the temperature optimum of photosynthesis (Hatfield et al. 2011) and therefore, greater [CO₂] response of crop yield at high temperatures may not occur.

The benefit from elevated [CO₂] is expected to be greater in times of lower water availability because stomatal conductance often decreases at elevated [CO₂] (Easterling et al. 2007; Ainsworth & Rogers 2007; Leakey et al. 2009a). In the absence of large changes in leaf area index (LAI) at elevated [CO₂] (Ainsworth & Long 2005), a decrease in stomatal conductance can increase soil moisture and canopy water use efficiency (Leakey et al. 2009a; Hussain et al. 2012). The increased water use efficiency associated with elevated [CO₂] is especially beneficial under drought conditions, since it allows plants to avoid excessive water loss for a longer time. This has been observed in C₄ species which have no direct stimulation in growth or photosynthesis at elevated [CO₂] due to their CO₂ concentrating mechanism, but can have a stimulation in growth when water is limiting due to the increase in water use efficiency (Ottman et al. 2001; Markelz et al. 2011; van der Kooi et al. 2016).

In addition to variation in [CO₂] response due to environmental conditions, there is also considerable variation in responses to elevated [CO₂] across different species and even genotypes within species (Ainsworth & Long 2005; Hasegawa et al. 2013; Bishop et al. 2014, 2015), although FACE and open top chamber experiments have investigated a limited number of plant species (Fig. 1.3). Determining what drives some of this inter- and intra-specific variation could inform future improvements in yield, since global [CO₂] continues to increase and traditional
breeding has not selected for greater responsiveness to elevated [CO₂] (Manderscheid & Weigel 1997; Ainsworth et al. 2008; Ziska et al. 2012). As global human population increases, current trends in crop improvement are expected to be insufficient to meet demands (Ray et al. 2013), thus alternative methods of increasing yield will become ever more important (Ainsworth et al. 2008; Ziska et al. 2012).

Previous experiments have demonstrated that sink strength is an important component of intraspecific variation in response to elevated [CO₂] (Ziska et al. 2001; Hasegawa et al. 2013; Aranjuelo et al. 2013). In rice, sink capacity, defined as the product of spikelet density and single-grain mass, was positively correlated with yield response to elevated [CO₂] in a field setting (Hasegawa et al. 2013). Stimulation in panicle density and seeds per panicle made the largest contribution to the yield stimulation at elevated [CO₂]. Harvest index, or grain mass divided by total aboveground biomass, was associated with increased response to elevated [CO₂] in wheat (Aranjulelo et al. 2013). A similar comparison of cultivars to determine the role of sink capacity and other factors in the response to elevated [CO₂] of soybean in the field has not yet been performed. Soybean is the fourth most produced crop and the most important oilseed crop in the world (Ainsworth et al. 2012). Determining cultivar variation in yield response to elevated [CO₂] and the traits associated with it would be an important first step in maximizing [CO₂] response in this crop.

The link between sink capacity and response to elevated [CO₂] is hypothesized to occur because greater photosynthetic C assimilation at elevated [CO₂] leads to excess sugar production, which cannot be immediately exported to sink tissues, and therefore accumulates in leaf mesophyll cells (Krapp & Stitt 1995). The excess sucrose can be cycled through invertase and hexokinase and turned into hexoses. These hexoses are sensed by hexokinase, which can signal a decrease in Rubisco expression and decrease photosynthetic capacity (Moore et al. 1999; Sharkey et al. 2004). Phloem is the primary conduit of sugars from the leaf to sink tissue, and so the capacity the plant has to load the phloem with sugars and prevent buildup in the leaf could be an important regulator of sink capacity. Since sink capacity is related to response to elevated [CO₂], this suggests that phloem loading capacity could play a role in this response.

In order to maintain high concentrations of sugar in leaf phloem and facilitate mass flow of sugars from source to sink tissue, plants have evolved multiple strategies for loading phloem tissue (Rennie & Turgeon 2009). The three primary strategies are apoplastic loading, passive
loading, and polymer trapping. In apoplastic loading species, sucrose diffuses through mesophyll cells until it is exported to the apoplasm between the mesophyll and companion cell by SWEET transporters (Chen 2014). This sucrose is then imported into the companion cell by proton-sucrose symporters (SUT/SUC; Lalonde et al. 2004; Yadav et al. 2015). In passive loading species, plasmodesmatal connections exist between mesophyll and companion cells so sucrose can passively diffuse through without any concentrating step. This strategy therefore requires high mesophyll sucrose concentrations to maintain a concentration gradient. Polymer trapping species also have symplastic continuity between mesophyll and companion cells, but sucrose that diffuses into the companion cell is turned into the larger sugars raffinose and stachyose (Rennie & Turgeon 2009). Raffinose and stachyose are too large to diffuse through plasmodesmata, so the concentration gradient is maintained for more sucrose to diffuse.

Previous experiments have found a link between phloem loading strategy and response to short term changes in carbon supply (Körner et al. 1995; Amiard et al. 2005; Adams et al. 2007). When apoplastic loading and polymer trapping plants were transferred from low light to high light, the apoplastic loading plants were able to substantially increase their photosynthetic and phloem loading capacity while the polymer trapping species accumulated sugars in the mesophyll cells and showed little increase in photosynthesis after the transfer to high light (Amiard et al. 2005; Adams et al. 2007). These results suggest that the polymer trapping species are anatomically constrained since plasmodesmata are generally fixed by the end of leaf development, unlike sucrose transporter expression. When apoplastic, passive, and polymer trapping species were grown at ambient [CO₂] and then transferred to elevated [CO₂] for six to ten days, all species increased their total nonstructural carbohydrates (TNC; Korner et al. 1995). Passive and polymer trapping species, which were pooled together, generally had higher TNC than apoplastic loading species. No studies to date have been performed, however, comparing apoplastic and passive loading species at elevated [CO₂] across a full growing season.

In addition to phloem loading strategy, increasing phloem loading capacity has been suggested as a mechanism for increasing photosynthesis and yield, particularly in times of high source supply, such as at elevated [CO₂] (Ainsworth & Bush 2011; Stitt 2013). An increase in sucrose transporters could theoretically allow for more sucrose to be pumped out of the leaf at elevated [CO₂], therefore preventing sugar mediated downregulation of photosynthesis. Results from overexpressing sucrose transporters, however, have been mixed (Leggewie et al. 2003;
Dagupta et al. 2014; Wang et al. 2015). Wang et al. (2015) transformed the Arabidopsis thaliana sucrose transporter AtSUC2, driven by the companion cell specific promoter PP2, into rice. Yield was enhanced in the transgenic line and there was no change in photosynthesis or leaf soluble sugar content. In contrast, Dasgupta et al. (2014) overexpressed AtSUC1, AtSUC2, or the maize sucrose transporter ZmSUT1 in Arabidopsis thaliana using the viral companion cell specific promoter CoYMV. They observed a decrease in biomass and increase in soluble sugars in the leaves as well as an increase in gene expression related to phosphate limitation. Further studies will be needed to determine whether increasing sucrose transporter expression at elevated [CO2] will enhance response to elevated [CO2] or if a perceived phosphate limitation limits the benefit.

**Research objectives**
The aim of my thesis is to better understand the variation in C3 plant responses to elevated [CO2] due to environment, sink strength, and carbon allocation. Determining the drivers of this variation in [CO2] response can better inform model predictions of future climate and food supply and potentially assist breeding and transgenic efforts to maximize [CO2] response and consequently future yields. The first objective of my thesis was to determine how growing season temperature and water inputs impact crop responses to elevated [CO2] using the global dataset of OTC and FACE experiments. Most previous studies examining the interaction between these factors focus on a single species and one location. The study presented in Chapter 2 therefore uses meta-analysis of OTC and FACE experiments to test the prediction that environment impacts the response of yield and biomass to elevated [CO2] using growing season temperature and water input data.

Response to elevated [CO2] varies not only with environment, but also between species and cultivars. The second objective of my thesis was to determine the variation in yield response to elevated [CO2] across soybean cultivars grown in the field. The study presented in Chapter 3 uses data from 18 soybean cultivars grown in two years and nine of those cultivars grown in four years in a FACE experiment to determine the genetic variation in yield response to elevated [CO2] and the consistency of that response across multiple years. Sink strength is one of the drivers of plant responses to elevated [CO2], so this study also aimed to determine what
components of sink strength and plant architecture were related to a greater stimulation in yield from elevated [CO₂] in soybean.

The third objective of my thesis was to determine the role of phloem loading strategy in response to elevated [CO₂]. At elevated [CO₂] and when sink strength is low, sugars can build up in the leaf and downregulate Rubisco, therefore decreasing potential photosynthetic capacity (Moore et al. 1999). Therefore, the mechanism of sucrose transport to phloem may alter the potential buildup of sugars and decrease in photosynthetic capacity at elevated [CO₂]. Plants have evolved different mechanisms for loading phloem with sugar and the study presented in Chapter 4 compared plants with two different strategies: apoplastic and passive loading. This experiment compared the response to elevated [CO₂] of two apoplastic loading species, pea (Pisum sativum) and beet (Beta vulgaris), and two passive loading species, strawberry (Fragaria x ananassa) and peony (Paeonia lactiflora) in a mini FACE field experiment in 2013 and 2014. Data presented in Chapter 4 aims to understand how these species differed in their photosynthetic, biochemical, and leaf anatomical responses to elevated [CO₂].

Phloem loading could have an impact on response to elevated [CO₂] not just through different mechanisms, but also in the plant’s capacity to load phloem. The fourth objective of my thesis was to determine how altering the expression of sucrose transporters used for loading phloem in Arabidopsis thaliana would impact the plant’s photosynthetic and biomass responses to elevated [CO₂]. Arabidopsis thaliana is primarily an apoplastic loading species which uses AtSUC2 to load sucrose into the phloem (Gottwald et al. 2000). Previous studies of plants overexpressing sucrose transporters have had mixed results (Dasgupta et al. 2014; Wang et al. 2015), with some showing an increase in biomass and others a decrease in biomass due to a perceived phosphate limitation. The study presented in Chapter 5 compares two transgenic lines with wild-type plants. In the HvSUT1 genotype, the native AtSUC2 was replaced with HvSUT1 driven by the AtSUC2 promoter (Reinders et al. 2012). HvSUT1 has a stronger affinity and transport activity for sucrose, so could enhance phloem loading. In the AtSUC1 genotype, another Arabidopsis sucrose transporter, AtSUC1, was expressed in a wild-type background using the viral, companion cell-specific promoter CoYMV (Dasgupta et al. 2014). By increasing sucrose transporter expression above native levels, phloem loading could be enhanced even more strongly than in the HvSUT1 genotype and response to elevated [CO₂] could be further stimulated. These two genotypes were tested against wild-type to determine whether plants with
altered sucrose transporter expression would have greater response to elevated [CO₂] due to less sugar-mediated downregulation of photosynthesis or would have little response to elevated [CO₂] due to a perceived phosphate limitation.
Figure 1.1. Global distribution of Free Air CO₂ Enrichment (FACE) and open top chamber (OTC) experiments on plants rooted in the ground. Taken from Leakey et al. (2012).
Figure 1.2. Frequency distribution of global land (based on 25 km² pixel cells) with given annual average precipitation (gray histograms in upper panel) and temperature (gray histograms in lower panel), averaged from 1950-2000, as extracted from the WorldClim dataset (Hijmans 2005). Frequency distributions of global open-top chamber (OTC) and free air CO₂ enrichment (FACE) experiments are superimposed. Taken from Leakey et al. (2012).
Figure 1.3. The distribution of OTC and FACE experiments across the angiosperm phylogeny (Angiosperm Phylogeny Group 2009). The number of species investigated in OTC and FACE experiments is shown in parentheses (OTC / FACE) followed by the total estimated number of species within each group. For example, 27 Poales species have been investigated in OTC experiments and 47 in FACE experiments out of an estimated 18,326 species in the order. Taken from Leakey et al. (2012).
CHAPTER II: HOW SEASONAL TEMPERATURE OR WATER INPUTS AFFECT THE RELATIVE RESPONSE OF C₃ CROPS TO ELEVATED [CO₂]: A GLOBAL ANALYSIS OF OPEN TOP CHAMBER AND FREE AIR CO₂ ENRICHMENT (FACE) STUDIES

Abstract

Rising atmospheric carbon dioxide concentration ([CO₂]) has the potential to positively impact C₃ food crop production by directly stimulating photosynthetic carbon gain (A), which leads to increased crop biomass and yield. Further stimulation of A and yield can result from an indirect mechanism in which elevated [CO₂] often decreases stomatal conductance and canopy water use, ameliorating drought stress. Experiments in Open Top Chambers (OTC) and Free Air CO₂ Enrichment (FACE) facilities have enabled investigation of crop responses to elevated [CO₂] in near natural, field conditions. Mechanistic understanding of physiological responses to elevated [CO₂] has led to predictions that the stimulation of A, biomass production and economic yield will vary with the temperature and water supply experienced by the crop. This study tested current assumptions about the relationships between relative responses of yield and biomass to elevated [CO₂] and variation in growing season temperature and water inputs (precipitation plus irrigation). Growing season average temperature was not a good predictor of the magnitude of biomass and yield responses to elevated [CO₂], contradicting the prediction that responses to elevated [CO₂] would increase with increasing temperature due to the greater benefit from decreasing photorespiration. However, the prediction that the relative stimulation of yield by elevated [CO₂] would be greatest in drier conditions was generally supported. Thus, a simple CO₂ fertilization value is not appropriate for modeling future crop productivity under varying environmental conditions. Further studies are necessary across a broader range of environmental conditions in order to accurately predict how rising [CO₂] will interact with temperature and drought stress and alter future crop production.

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Introduction
Atmospheric carbon dioxide concentration ([CO2]) has increased substantially since the Industrial Revolution, and will continue to increase given recent evidence that terrestrial and oceanic CO2 sinks are not growing at the same rate as anthropogenic CO2 emissions (Canadell et al. 2007; Le Quere et al. 2009). The increase in atmospheric [CO2] is the major contributing factor to global warming (Forster et al. 2007), but elevated [CO2] also directly stimulates light-saturated, net photosynthetic CO2 uptake (A) in C3 crops, generally leading to greater crop biomass production and yield (Kimball 1983; Kimball et al. 2002; Ainsworth & Long 2005). Understanding of crop responses to elevated [CO2] has been greatly improved by studies performed with crops rooted in the ground at farm field sites. Field experiments, including those done with Open Top Chambers (OTC) and Free Air CO2 Enrichment (FACE), allow for fumigation of plants in near natural conditions throughout the growing season. Although OTC and FACE experiments have been performed primarily in temperate regions of the Northern Hemisphere, they have provided a wealth of data on C3 crop responses to elevated [CO2] at a range of growing season temperatures and precipitation levels (Leakey et al. 2012).

Future increases in atmospheric [CO2] are predicted to be accompanied by increased growing season temperatures and altered precipitation events (Forster et al. 2007). These changes in temperature and water availability will likely alter C3 plant responses to elevated [CO2] (Long 1991; Morgan et al. 2004; Ziska et al. 2012). Theoretically, the response of C3 photosynthesis to elevated [CO2] is predicted to be greater at higher temperatures because the increase in [CO2] can counteract greater rates of Rubisco oxygenation and subsequent photorespiration at higher temperatures (Long 1991). This theory is supported by the observation that the relative CO2 response of diurnal carbon uptake in soybean increased with daily maximum temperature (Bernacchi et al. 2006). However, there is less direct evidence that the relative CO2 stimulation of C3 crop biomass or yield is greater at higher temperatures, and in fact, a synthesis of rice FACE experiments suggests that the relative CO2 response of rice yields decreases with increasing growing season temperature (Hasegawa et al. 2013). This may be because the optimum temperature for C3 crop photosynthesis is not always the same as the optimum temperature for C3 crop yield (Hatfield et al. 2011), and higher temperatures can have a more negative impact on reproductive processes than on photosynthesis (Fuhrer 2003; Prasad et al. 2003; Welch et al. 2010). A field study with soybean which analyzed the combined effects of
elevated [CO₂] and temperature found greater relative stimulation by elevated [CO₂] in daily carbon uptake at elevated temperature across two years, but greater stimulation in biomass and seed yield was only apparent during one of the years (Ruiz-Vera et al. 2013). The second year was warmer than average during the middle of the growing season, so the increased stimulation of photosynthesis at elevated temperature may not have resulted in increases in total biomass or yield when temperatures were high enough to affect processes such as reproductive viability or respiration.

In times of lower water availability, crops are expected to show a greater relative response of A, biomass and yield to elevated [CO₂] (Easterling et al. 2007; Ziska et al. 2012). This occurs because elevated [CO₂] often decreases stomatal conductance (gₛ) (Ainsworth & Rogers 2007), and in the absence of large changes in leaf area index (LAI; Ainsworth & Long 2005), the crop canopy then uses less water and soil moisture availability is greater (Leakey et al. 2009a; Hussain et al. 2013). Therefore, in times or places with limited water availability, a crop growing at elevated [CO₂] can avoid the negative effects of drought for a longer period of time than a crop growing at ambient [CO₂]. In FACE experiments, this has been demonstrated in sorghum and maize, both C₄ species that do not show any direct stimulation of A or LAI at elevated [CO₂] (Ottman et al. 2001; Markelz et al. 2011). In the same way, C₃ wheat showed a greater relative stimulation of yield in dry compared to irrigated plots (Kimball et al. 1995). Historical crop yield data is also consistent with an increase in CO₂ response with decreasing water availability in both C₄ and C₃ crops (McGrath & Lobell 2011).

Currently, predictions of crop biomass and yield response to elevated [CO₂] assume that these physiological mechanisms operate consistently across all climates. Analyses of interannual variation in crop response to elevated [CO₂] at individual sites have both supported (Bernacchi et al. 2006) and challenged (Hasegawa et al. 2013) this assumption. This study extends the analysis to a larger dataset from a global network of FACE and OTC experiments that included all the major C₃ crops and a broader range of climatic conditions. Previous global analyses of FACE and OTC experiments have considered the average responses of species to elevated [CO₂], but have not considered climate as a predictive factor for the magnitude of biomass and yield responses to elevated [CO₂] (Ainsworth & Long 2005). Two broad predictions are tested in the current study: (1) the relative stimulation of C₃ crop above-ground biomass and economic yield by elevated [CO₂] is positively correlated to average growing
season temperature and (2) the relative stimulation of C3 crop above-ground biomass and economic yield by elevated [CO2] is negatively correlated to total growing season water supply.

**Materials and Methods**

*Database Compilation*

A database of articles reporting C3 crop responses to elevated [CO2] from FACE and OTC experiments was created for a previous analysis (Leakey et al. 2012). Additional papers that reported crop responses to elevated [CO2] were identified by searching the ISI Web of Knowledge database (Thomson ISI, Philadelphia, PA, USA). Studies of C3 food crop responses to elevated [CO2] in OTC or FACE experiments where plants were rooted in the ground were included in the database if they reported A, economic yield (i.e., seed or tuber weight), biomass at maturity or harvest index. Parameter values for different genotypes or CO2 treatments were assumed to be independent, therefore included separately in the database, following the methods of previous meta-analyses (Curtis & Wang 1998; Medlyn et al. 1999; Ainsworth et al. 2002).

The mean value at ambient and elevated [CO2], standard deviation of the mean and sample size for each variable were taken from tables or extracted from figures using digitizing software (Grafula 3 v.2.10, Wesik SoftHaus, St. Petersburg, Russian Federation).

Growing season temperature (24 hr mean), precipitation, and irrigation values were obtained from the primary literature, personal communication, or the iAIMS Climatic Database (https://beaumont.tamu.edu/CLIMATICDATA/WorldMap.aspx?index=WorldMap). Preference was given in that order. Growing season was assumed to occur from planting to harvest, excluding dormant periods when appropriate as, for example, in winter wheat.

*Meta-Analysis*

Data from 18 OTC sites and 11 FACE sites (Table 2.1) reported in 72 primary manuscripts (Appendix A) were used for the analyses. Because OTC experiments were done at a wide range of elevated [CO2], this analysis was limited to studies with an elevated treatment of 600 to 750 ppm (Table 2.1), since this was the concentration range with the greatest number of primary studies. Because the mean treatment concentration for OTC studies was greater than the CO2 concentration used in FACE experiments, OTC and FACE studies were analyzed separately. The meta-analysis was restricted to the highest nitrogen or phosphorus treatment and ambient
ozone in order to minimize the interactive effects of those stresses on the interpretation of the CO$_2$ response. The response ratio ($r =$ response in elevated [CO$_2$]/response in ambient [CO$_2$]) was used for all analyses. A mixed effects model was used based on the assumption of random variation in responses among studies. Many of the studies did not report the standard deviation of the means and so an unweighted analysis was performed following previous methods (Gurevitch & Hedges 1999; Leisner & Ainsworth 2011). Effects were considered significant when the 95% confidence interval did not span 0 (Curtis & Wang 1998). Effects between species were considered significant when the 95% confidence intervals did not overlap. The C$_3$ crop average response reported in Figures 2.1 and 2.2 includes data from all species. However, at least 3 degrees of freedom were required for a species-specific estimate to be reported.

**Regression Analysis**

Linear regression was used to test the association between CO$_2$ response ratio ($r$) for above-ground biomass or economic yield and growing season temperature or water availability (growing season precipitation + irrigation). If more than one genotype or cultivar of crop was grown at a single location in a single year, the data were averaged so that one data point in the regression analysis represented a single year, species, and site. Regression analysis was performed using the regression procedure (Proc REG) with SAS (SAS Institute, Cary, NC, USA). Data describing elevated CO$_2$ impacts on $A$ were excluded from regression analyses because it is highly dependent on leaf temperature during measurements, which was highly variable and often not reported. Potato, sugar beets, and other root or tuber crops were analyzed separately because these species have fundamentally different patterns of carbon allocation above- and below-ground. There were not enough observations of tuber crop species from FACE experiments to perform regression analyses.

**Results**

**CO$_2$ Responses**

Growth at elevated [CO$_2$] significantly increased $A$, biomass production and economic yield for C$_3$ crops in both FACE (Fig. 2.1) and OTC experiments (Fig. 2.2). Eight C$_3$ crop species have been investigated at elevated [CO$_2$] in FACE experiments and seven species have been investigated in OTC experiments (Table 2.1). The average elevated [CO$_2$] in FACE experiments
was 560 ppm, with a range of 545-625 ppm. For OTC experiments, the average was 691 ppm, with a range of 600-740 ppm. Due to the difference in CO$_2$ concentration, the two datasets were not combined. In both FACE and OTC experiments, the average relative stimulation in light-saturated photosynthetic rate was greater than the stimulation in biomass production or economic yield (Figs. 2.1 & 2.2), consistent with earlier analyses (Long et al. 2006). This may reflect the fact that rates of nighttime respiration in leaves can also be greater at elevated [CO$_2$] (Davey et al. 2004; Leakey et al. 2009a; Fukayama et al. 2011; Markelz et al. 2014a).

This study investigated drivers of variation in C$_3$ crop responses to elevated [CO$_2$] and tested if different species showed different responses to elevated [CO$_2$] in FACE and OTC experiments. In the FACE experiments, *Manihot esculenta* (cassava) had the greatest relative stimulation in photosynthesis at elevated [CO$_2$] (Fig. 2.1a; Table 2.2), although it was not significantly greater than wheat (Fig. 2.1a). The single measurement of cassava yield response to elevated [CO$_2$] reported an 89% increase in tuber fresh weight at elevated [CO$_2$] (Rosenthal et al. 2012). Other C$_3$ crops, *Glycine max* (soybean), *Oryza sativa* (rice) and *Triticum aestivum* (wheat), showed similar responses to elevated [CO$_2$], with overlapping confidence intervals among the species in the relative CO$_2$ response of $A$, biomass and yield measured at FACE experiments (Fig. 2.1; Table 2.2).

There was significant variation among species in their photosynthetic, biomass and yield responses to elevated [CO$_2$] reported in OTC experiments. *Glycine max* (soybean) showed no stimulation in $A$ at elevated [CO$_2$] (Fig. 2.2a), but significant stimulation in biomass and yield (Fig. 2.2b, c). *Solanum tuberosum* (potato) did not show any relative increase in above-ground biomass at elevated [CO$_2$] (Fig. 2.2b), but showed significant stimulation in tuber yield, comparable to the seed yield response of other species (Fig. 2.2c; Table 2.2). Notably, the 95% confidence intervals for all species responses to elevated [CO$_2$] were wide, supporting the hypothesis that genotypic variation and/or environmental variation also significantly affects the CO$_2$ response of C$_3$ crops.

**CO$_2$ x Temperature Interaction**

In contrast to the first hypothesis that the relative response of C$_3$ crops to elevated [CO$_2$] would be greater at higher temperatures, there was no correlation between average temperature and the relative stimulation of above-ground biomass by growth at elevated [CO$_2$] in FACE (Fig. 2.3a).
There was also no significant correlation between C₃ crop yield response and growing season temperature in either FACE or OTC experiments (Fig. 2.3c, d). Only the stimulation of aboveground biomass by growth at elevated [CO₂] in OTC experiments responded as predicted with significantly greater stimulation of biomass production associated with greater temperatures (p = 0.010) (Fig. 2.3b). This trend in aboveground biomass CO₂ response was accompanied by a significant decrease in the relative effect of CO₂ on harvest index with increasing growing season temperature in OTC (Fig. 2.3f). There was no equivalent response of harvest index in FACE experiments (Fig. 2.3e).

There is significant variation among species in the average growing season temperature, and these differences may make it more difficult to determine CO₂ x temperature trends within a species. Because wheat has been grown at elevated [CO₂] in 6 FACE experiments on 4 continents and 9 OTC experiments, its relative CO₂ response was investigated independently from other species. Again, there was no trend between relative yield or biomass response and temperature, either in FACE or in OTC experiments (Fig. 2.4).

CO₂ x Water Input Interaction

Consistent with the second hypothesis, yield response in both FACE and OTC experiments was significantly negatively correlated with water input (growing season precipitation + irrigation) (Fig. 2.5c, d). This trend, however, was not observed in above-ground biomass (Fig. 2.5a, b). This disparity between biomass and yield response did not correspond to a change in harvest index response (Fig. 2.5e, f). The range of water availability conditions was greater in OTC experiments than in FACE experiments, due to a larger number of irrigated experiments, although the trends were similar in data from both experimental approaches. Many OTC experiments also did not report their irrigation, so there were fewer observations to use for the regression analysis with water input compared to temperature.

When wheat and potato were analyzed separately, the change in yield and biomass responses with respect to water input was still observed. For both FACE and OTC experiments, wheat yield response significantly decreased with increasing water availability (Fig. 2.6c, d). Aboveground biomass response, however, did not respond to [CO₂] (Fig. 2.6a, b). Across OTC experiments, (sweet) potato tuber biomass response and yield response decreased significantly with increasing water availability (Fig. 2.7). These species- and growth habit-specific results,
like the overall yield response results, agreed with the initial prediction that biomass and yield
[CO₂] response would be greatest in dry conditions.

Discussion
It has been widely reported that elevated [CO₂] stimulates A, above-ground biomass production
and the economic yield of C₃ species (Figs. 2.1, 2.2; Kimball 1983; Lawlor & Mitchell 1991;
Jablonski et al. 2002; Kimball et al. 2002; Ainsworth & Long 2005; Long et al. 2006). Meta-
analyses have shown that C₃ crops have greater reproductive responses to elevated [CO₂] than
wild species (Jablonski et al. 2002), and that there is significant regional variation in the
magnitude of the CO₂ fertilization effect (McGrath & Lobell 2013). Across all FACE and OTC
experiments, the mean relative economic yield response to elevated [CO₂] does not differ
significantly among C₃ crop species (overlapping confidence intervals in Figs. 2.1c, 2.2c).
However, there is significant variation in the magnitude of stimulation of biomass and yield
casted by growth in elevated [CO₂], indicated by the wide confidence intervals in Figs 2.1 and
2.2. A number of factors likely cause that variation, including genotypic differences in CO₂
responsiveness (e.g., Yang et al. 2006; De Costa et al. 2007; Leakey & Lau 2012; Ziska et al.
2012) and/or interactions between environmental factors and elevated [CO₂] (Porter & Semenov
2005; McGrath & Lobell 2013). Using this global dataset of FACE and OTC experiments, we
investigated whether seasonal temperature or water inputs were predictive of the relative
response of C₃ crops to elevated [CO₂].

Because Rubisco oxygenation reactions and subsequent photorespiration rates increase
with increasing temperature (Jordan & Ogren 1984), the theoretical response of C₃
photosynthesis to elevated [CO₂] is greater at high temperatures (Long 1991). Therefore, it has
been hypothesized that the relative CO₂ response of biomass and yield will be positively
related to growing season temperature. This assumption has been applied in modeling
studies to predict much larger stimulation of plant biomass production by elevated CO₂ in hot
versus cold climates (Hickler et al. 2008). However, the meta-data in the current study do not
support the hypothesis (Fig. 2.3), and there is no correlation between growing season
temperature and crop biomass or yield except in biomass at OTC experiments. A recent meta-
analysis summarizing experimental reports of plant responses to growth at elevated [CO₂] and
elevated temperatures also showed that the CO₂ response of photosynthesis in C₃ species was
greater when plants were grown at elevated temperatures (defined as 1.4–6.0 °C above ambient) or under heat stress (defined as >8.0 °C above ambient; Wang et al. 2012). However, as in this analysis, the greater relative [CO₂] response of photosynthesis at elevated temperatures or heat stress did not necessarily translate into a greater relative response of biomass production (Wang et al. 2012).

The lack of correlation between the magnitude of yield response to elevated [CO₂] and growth temperature may result from a number of responses to high temperature stress that could prevent the benefits of enhanced photosynthetic carbon gain from being realized at high temperatures. First, temperature effects on respiration do not always balance the effects on photosynthesis (Atkin et al. 2005; Piao et al. 2008). Second, high temperatures can damage reproductive processes independently from atmospheric [CO₂] (Prasad et al. 2003; Caldwell et al. 2005, Hasegawa et al. 2013; Ruiz-Vera et al. 2013) and the optimum temperature for vegetative growth is often higher than the optimum temperature for reproductive growth in crops (Hatfield et al. 2011). Third, high temperatures also increase transpiration and demand for soil moisture (Lobell et al. 2013), making the interactive effects of elevated [CO₂] and temperature intrinsically coupled to water availability. Interestingly, in the subset of experiments reviewed in this study that reported precipitation and temperature, there is a significant positive correlation between growing season temperature and precipitation (Fig. 2.8). The increase in precipitation with increasing temperatures across both FACE and OTC experiments may have modified the response to elevated [CO₂] at higher temperatures. Average growing season temperatures also varied for different species, such that wheat grew at cooler temperatures than soybean or rice (Table 2.1). It is also possible that differences among species in [CO₂] response made it more difficult to determine interactions between temperature and elevated [CO₂].

An important distinction between the meta-analysis of Wang et al. (2012) and this study is that the meta-data presented here are for diverse species and genotypes of crops in their normal growing regions. Therefore, the crops were adapted to the conditions in which they were being grown, and while some years are warmer or cooler than average, they generally represent “ambient” growing conditions. This might be expected to diminish the extent to which crops grown under warmer conditions experienced stress. For example, the rice varieties grown at Wuxi, China with a mean growing season temperature of 23.9 °C were locally adapted, and might not be expected to experience stress relative to the cooler-climate adapted varieties grown
at Shizukuishi, Japan with a mean growing season temperature of 19.3 °C. This would reduce the
likelihood of problems with reproductive failure. In addition, when plants are grown in the long-
term at higher temperatures, acclimation of respiration tends to moderate CO₂ losses (Atkin et al.
2005). Instead, this might suggest that processes inherently linked to greater temperatures,
especially greater water use, are playing a more important role in offsetting CO₂ effects on
photosynthesis.

The second hypothesis was that growing season water supply would be negatively
correlated to the CO₂ response of above-ground biomass production and economic yield.
Elevated [CO₂] reduces stomatal conductance (Curtis 1996; Drake et al. 1997; Ainsworth &
Rogers 2007), which can lead to decreases in canopy evaportranspiration of crops (Hunsaker et al.
2000; Yoshimoto et al. 2005; Hussain et al. 2013), greater soil moisture availability (Burkart
et al. 2011) and the ability to withstand mild drought stress (Wall et al. 2006). This analysis of
C₃ economic yield supported this hypothesis and there was a negative correlation between
relative CO₂ response of yield and seasonal water input in both OTC and FACE experiments
(Fig. 2.4c, d). This trend was also apparent when the species that have been studied most
intensively were evaluated independently. Wheat yield was negatively correlated with water
availability in both FACE and OTC experiments (Fig. 2.6c, d), and (sweet) potato tuber yield
and aboveground biomass were significantly negatively correlated with water availability in
OTC experiments (Fig. 2.7). These findings are consistent with attempts to assess the importance
of CO₂ fertilization effects to historical yield trends by contrasting yield response in wet and dry
years (McGrath & Lobell 2011). It is important to note that this analysis only showed the
relative response of crops to elevated [CO₂] and that the absolute magnitude of crop yields will
be much less under drought stress (Farooq et al. 2009). A major limitation of the dataset in the
present study was the lack of information for water inputs; in particular, the quantity of irrigation
was often not reported in OTC and FACE experiments. Consequently, there was less data to use
in the water availability analysis compared to the temperature analysis. This dataset, however,
was large enough to demonstrate that across the global dataset of crop responses to elevated
[CO₂], water availability plays a significant role determining the degree of yield response. This
interaction should be taken into account when modeling expected increases in yield with globally
increasing [CO₂].
This analysis used seasonal average temperature and total water input trends; therefore, the roles that extreme climatic events, daytime versus nighttime temperatures, and the timing of heat or drought stress events played in determining the relative CO₂ response were not considered. Climate variability and the frequency of extreme events affect yield potential and yield stability, and can also impact the relative response of crops to elevated [CO₂] (Porter & Semenov 2005). Prolonged heat waves and drought stress drastically reduce ecosystem gross primary productivity (Ciais et al. 2005) and crop production (Boyer 1982; Chaves & Oliveira 2004; Lobell et al. 2012). Both droughts and heat waves will likely become more frequent with global change and are also projected to be more common in the future during the period of reproductive growth (Gourdji et al. 2013), which is the most critical period for economic yield. It would be interesting to try to repeat this analysis with information about heat waves or drought events, but the data to do so are currently lacking, and additional manipulative experiments that test the interaction of rising [CO₂] and extreme events are needed.

**Conclusions**

Two key uncertainties about C₃ crop responses to elevated [CO₂] are how the relative stimulation of biomass and economic yield will vary with rising temperatures and drought stress (Easterling et al. 2007). Accurate projections of future crop productivity and food security critically depend upon successful understanding and mathematical simulation of the effects of elevated [CO₂] on crop plants (Parry et al. 2004; Müller et al. 2010; Asseng et al. 2013). This study presents the global dataset of C₃ food crop responses to elevated [CO₂] in field studies, which is an important resource for testing model performance under elevated [CO₂] across diverse growing conditions (Tubiello & Ewert 2002; Easterling et al. 2007; Asseng et al. 2013; Osborne et al. 2013). It demonstrates that on large scales, the stimulation of biomass production and economic yield by elevated [CO₂] rises as water inputs drop. It is notable that temperature has been argued to be a more important predictor of agricultural responses to climate change than precipitation (Lobell & Burke 2008), but under future elevated [CO₂], variation in water supply might be the more important factor. Other environmental factors such as nutrient supply and air pollution also are likely to influence the CO₂ response. Ultimately, more multi-factorial experiments coupled with modeling analyses are needed to improve understanding of C₃ crop responses to climate change, especially at the extremes of heat and drought stress which are likely to characterize future
growing environments. Particular effort is required to improve reporting of climate conditions and irrigation applied in these experiments to ensure they can be used in these sorts of analyses.
### Table 2.1. Description of the FACE and OTC sites and species used in this analysis. Symbols in the first column represent the site and species shown in Figs 2.3-2.8. Average growing season temperature and precipitation values from 1950-2000 were extracted from the WorldClim dataset (Hijimans et al. 2005).

<table>
<thead>
<tr>
<th>Site</th>
<th>Years of Experiment</th>
<th>C3 Species Studied</th>
<th>Elevated CO₂ Concentration (ppm)</th>
<th>Growing Season</th>
<th>Average GS Temperature (°C)</th>
<th>Average GS Precipitation (mm)</th>
</tr>
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<tbody>
<tr>
<td><strong>FACE</strong></td>
<td></td>
<td></td>
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<td>Maricopa, AZ, USA</td>
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<td>550-570</td>
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<td>May-Oct</td>
<td>19.6</td>
<td>554</td>
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<tr>
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<td>590</td>
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<td>22.0</td>
<td>384</td>
</tr>
<tr>
<td></td>
<td>2001-2004</td>
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<td>550</td>
<td>May-Sep</td>
<td>15.3</td>
<td>309</td>
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<td>Stuttgart, Germany</td>
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<td>525</td>
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<td>2007</td>
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<td>Nov-May</td>
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<tr>
<td>Yangzhou, China</td>
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<td>Jun-Oct</td>
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<td>Shizukuishi, Japan</td>
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<td>Soybean</td>
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Table 2.1 (continued).

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<th>Location</th>
<th>Year 1</th>
<th>Crop</th>
<th>Yield</th>
<th>Season</th>
<th>Year 2</th>
<th>Crop</th>
<th>Yield</th>
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<td>Soybean</td>
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<td>Potato</td>
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<td>1998</td>
<td></td>
<td></td>
<td>Mar-Jul</td>
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<tr>
<td>Raleigh, NC, USA</td>
<td>1999-2004</td>
<td>Soybean</td>
<td>700</td>
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<td>2003</td>
<td>Peanut</td>
<td>730</td>
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<td>Potato</td>
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<td>May-Sep</td>
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<tr>
<td></td>
<td>1995-1996</td>
<td>Wheat</td>
<td>675/730</td>
<td>Apr-Jul</td>
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<tr>
<td>Braunschweig, Germany</td>
<td>1994-1999</td>
<td>Wheat</td>
<td>680</td>
<td>Apr-Aug</td>
<td></td>
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<td></td>
<td>1995-1996</td>
<td>Potato</td>
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<td>May-Aug</td>
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<td>Jun-Sep</td>
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<td>700</td>
<td>May-Aug</td>
<td>1998</td>
<td>Peanut</td>
<td>730</td>
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Table 2.2. Results of the meta-analysis on photosynthesis ($A$), aboveground biomass, and economic yield in FACE and OTC experiments. The mean effect size and the lower and upper 95% confidence intervals (CI) are reported. df: degrees of freedom

<table>
<thead>
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Figure 2.1. Mean response to elevated [CO$_2$] (+/- 95% confidence intervals) of photosynthesis (a), aboveground biomass (b), and economic yield (c) of different C$_3$ crop species in FACE experiments. A description of the FACE sites is provided in Table 2.1. df: degrees of freedom
Figure 2.2. Mean response to elevated [CO₂] (+/- 95% confidence intervals) of photosynthesis (a), aboveground biomass (b), and economic yield (c) of different C₃ crop species in OTC experiments. A description of the OTC sites is provided in Table 2.1. df: degrees of freedom
Figure 2.3. Linear regression of growing season temperature versus the response ratio (elevated [CO₂] / ambient [CO₂]) of biomass (a, b), yield (c, d), and harvest index (e, f) in FACE (a, c, e) and OTC experiments (b, d, f). Each data point represents one species in one year at one location. Symbols are described in Table 2.1. Solid lines indicate statistically significant relationships (p < 0.05).
Figure 2.4. Linear regression of growing season temperature versus the response ratio (elevated [\text{CO}_2] / ambient [\text{CO}_2]) of wheat biomass (a, b) and yield (c, d) in FACE (a, c) or OTC experiments (b, d). Symbols are described in Table 2.1. There were no statistically significant relationships ($p < 0.05$).
Figure 2.5. Linear regression of growing season water availability (precipitation + irrigation) versus the response ratio (elevated [CO2] / ambient [CO2]) of biomass (a, b), yield (c, d), and harvest index (e, f) in FACE (a, c, e) and OTC experiments (b, d, f). Each data point represents one species in one year at one location. Symbols are described in Table 2.1. Solid lines indicate statistically significant relationships (p < 0.05).
Figure 2.6. Linear regression of growing season water availability (precipitation + irrigation) versus the response ratio (elevated [CO₂] / ambient [CO₂]) of wheat biomass (a, b) and yield (c, d) in FACE (a, c) or OTC experiments (b, d). Symbols are described in Table 2.1. Solid lines indicate statistically significant relationships (p < 0.05).
Figure 2.7. Linear regression of growing season water availability (precipitation + irrigation) versus the response ratio (elevated [CO₂] / ambient [CO₂]) of (sweet) potato tuber aboveground biomass (a) or yield (b) in OTC. Symbols are described in Table 2.1. Solid lines indicate statistically significant relationships (p < 0.05).
Figure 2.8. Linear regression of growing season temperature versus precipitation + irrigation (a, b) or precipitation (c, d) in FACE (a,c) and OTC (b, d). Solid lines indicate statistically significant relationships (p < 0.05). Symbols are described in Table 2.1.
CHAPTER III: IS THERE POTENTIAL TO ADAPT SOYBEAN (*GLYCINE MAX MERR.* ) TO FUTURE [CO\(_2\)]? AN ANALYSIS OF THE YIELD RESPONSE OF 18 GENOTYPES IN FREE AIR CO\(_2\) ENRICHMENT\(^2\)

Abstract
Rising atmospheric [CO\(_2\)] is a uniform, global change that increases C\(_3\) photosynthesis and could offset some of the negative effects of global climate change on crop yields. Genetic variation in yield responsiveness to rising [CO\(_2\)] would provide an opportunity to breed more responsive crop genotypes. A multi-year study of 18 soybean genotypes was done to identify variation in responsiveness to season-long elevated [CO\(_2\)] (550 ppm) under fully open-air replicated field conditions. On average across 18 genotypes, elevated [CO\(_2\)] stimulated total above-ground biomass by 22%, but seed yield by only 9%, since most genotypes showed a reduction in partitioning of energy to seeds. Over four years of study, there was consistency from year to year in the genotypes that were most and least responsive to elevated [CO\(_2\)], suggesting genetic control of CO\(_2\) response. Further analysis of six genotypes did not reveal a photosynthetic basis for the variation in yield response. Although partitioning to seed was decreased, cultivars with the highest partitioning coefficient in current [CO\(_2\)] also had the highest partitioning coefficient in elevated [CO\(_2\)]. The results show the existence of genetic variation in soybean response to elevated [CO\(_2\)], which is needed to breed soybean to the future atmospheric environment.

Introduction
For 20 million years before the Industrial Revolution, CO\(_2\) concentration ([CO\(_2\)]) was below 300 ppm and averaged just 245 ppm over the past 0.5 million years (Pearson & Palmer 2000; Barnola et al. 2003). The progenitors of our modern crops evolved under these conditions of [CO\(_2\)]. Since the Industrial Revolution, [CO\(_2\)] has increased from ~280 ppm in 1860 to ~400 ppm in 2013, and if current trends in emissions continue, atmospheric [CO\(_2\)] will exceed 500 ppm by 2050 (Ciais et al. 2013). For species with an annual growth cycle, it is highly unlikely that they

could adapt to this rapid rate of change (Leakey & Lau 2012). Elevated [CO₂] directly increases light-saturated, net photosynthetic CO₂ uptake (A) in C₃ plants by increasing [CO₂] at the site of Rubisco, and consequently increasing the velocity of carboxylation and decreasing the competitive oxygenation reaction that leads to photorespiration (Drake et al. 1997; Leakey et al. 2009a). Under light-limiting conditions, net CO₂ uptake is also increased because CO₂ inhibits the oxygenation reaction of Rubisco and allows a greater proportion of the limiting supply of ATP and NADPH to be used in photosynthetic carbon assimilation (Long & Drake, 1991). As a result, increasing [CO₂] is expected to increase photosynthesis in leaves under all lighting conditions and at all positions in the canopy. Improved photosynthesis at elevated [CO₂] typically leads to an increase in crop biomass and yield (Kimball 1983; Kimball et al. 2002; Long et al. 2004; Ainsworth & Long 2005), although there will likely be important regional differences in crop responses to elevated [CO₂] according to interactions with variation in climate and soil conditions (McGrath & Lobell 2013). Maximizing crop response to elevated [CO₂] could, at least in part, offset the damaging effects on yield of other aspects of global change, such as rising temperature, increased frequency and intensity of droughts, and increased vegetation to atmosphere water vapor pressure deficit (Ciais et al. 2005; Lobell & Field 2007; Lobell et al. 2014; Ort & Long 2014).

Soybean provides more than half of the world’s oilseed and is the world’s fourth most important crop in terms of seed production (Ainsworth et al. 2012). Soybean physiological responses to elevated [CO₂] have been extensively studied in both controlled environments and the field (reviewed by Ainsworth et al. 2002; Leakey et al. 2009a). In the Soybean FACE (SoyFACE) experiments, soybean showed a sustained increase in A and reduction in stomatal conductance (gs) when exposed to elevated [CO₂] (550 ppm) across multiple growing seasons (Rogers et al. 2004; Bernacchi et al. 2006). Increased A led to increases in leaf carbohydrate content (Ainsworth et al. 2004; Rogers et al. 2006), respiration (Davey et al. 2004; Leakey et al. 2009b), biomass production and seed yield (Morgan et al. 2005).

Although soybean has been well-characterized in open air field conditions, almost all of the SoyFACE experiments have been performed with a single cultivar, and in order to adapt soybean to elevated [CO₂], genotypic variation in yield responses is required. In controlled glasshouses, Ziska et al. (1998; 2001) investigated the yield response of nine soybean genotypes to elevated [CO₂], and reported significant variation in the magnitude of the yield response. The
basis of these increases varied with genotype. Some genotypes increased branching while others increased individual seed weight with growth at elevated [CO₂] (Ziska et al. 1998). These were important findings, but outside of the crop’s normal growing environment. A key further question is whether such variation can be found in a field setting and if greater variation might be identified with a larger panel of genotypes.

The SoyFACE facility provided a unique opportunity to assess responsiveness under open-air conditions in a major soybean production setting (Rogers et al. 2004). The large size of each elevated [CO₂] plot provides sufficient area to simultaneously test several cultivars at plot scales, replicated across the four blocks of the experimental facility (Rogers et al. 2004; Morgan et al. 2005). In this study, the yield response to elevated [CO₂] in 18 soybean genotypes was investigated, with 6 genotypes studied across 5 consecutive years. The objective of the study was to test if significant intraspecific variation in the yield response of soybean could be found under open-air field conditions, and to identify parameters correlated with the maximum yield response to elevated [CO₂].

Materials and Methods

Experimental Site

Soybean genotypes were grown at the SoyFACE facility in Champaign, IL, USA (40°02′N, 88°14′W, 228 m above sea level; http://www.igb.illinois.edu/soyface/). This facility has been described in detail previously (Ainsworth et al. 2004; Rogers et al. 2004). Briefly, SoyFACE is located on 32 ha of farmland where soybean and maize (Zea mays) are each planted on 16 ha of the facility, rotated annually. The field was tile drained and not irrigated, and the soil type is a Drummer-Flanagan. The experiment was conducted as a randomized complete block design (n=4) from 2004 to 2008. Environmental conditions, planting and harvesting dates in each year are provided in Table 3.1. Each block of the experiment consisted of two 20-m diameter octagonal plots separated by 100 m, with one plot maintained at current ambient [CO₂] (~380-390 ppm) and one plot fumigated with elevated [CO₂]. The target elevated [CO₂] was 550 ppm, which is the approximate concentration expected for the year 2050 (Ciais et al. 2013). Plots were fumigated with elevated [CO₂] during daylight hours from emergence to maturity, using the FACE system adapted from the design of Miglietta et al. (2001b). From 2004-2008, the average elevated [CO₂] ranged from 547 to 552 ppm, and one-minute averages were within ± 20% of the
Crop Growth and Yield

In 2004 and 2005, the yield response to elevated [CO₂] was investigated in 18 soybean genotypes, which were selected to test a variety of responses to elevated [CO₂]. Based on the responsiveness of those lines, nine genotypes were planted in every year from 2004 to 2007, A3127, Clark, Dwight, HS93-4118, IA3010, LN97-15076, Loda, NE3399 and Pana, and six genotypes were planted in 2008, Dwight, HS93-4118, IA3010, LN97-15076, Loda, NE3399 and Pana (Table 3.2). Developmental and yield traits were recorded using the center two rows of the genotype plots, with the outer rows serving as border rows. The traits were: total above-ground biomass at maturity (g plant⁻¹); average mass of 100 seeds (g); grain yield (kg ha⁻¹); partitioning coefficient (harvest index); lodging, scored as 1-10 at maturity with 1 representing all plants erect and 10 all plants prostrate; plant height, measured as the distance from the ground to the top node of the main stem (cm); and time to completion of maturity of ≥ 95% of the pods (R8), as judged by loss of chlorophyll. The partitioning coefficient was calculated based on energy content of vegetative biomass (18 MJ kg⁻¹) and seed biomass (23 MJ kg⁻¹) following Amthor et al. (1994).

Photosynthetic Gas Exchange

Midday leaf photosynthesis and transpiration of fully expanded leaves at the top of the canopy were measured on 14 July 2008 when the plants were in vegetative growth (stage 4; Fehr et al.)

3 Data collected by Randy Nelson
4 Data collected by Amy Betzelberger
1971) and on 31 July 2008 when the plants were flowering (stage R1; Fehr et al. 1971). Leaf photosynthetic CO₂ uptake and transpiration were measured with portable gas exchange systems incorporating infrared CO₂ and water vapor analyzers (LI-6400; Li-Cor, Lincoln, NE, USA) coupled with an integrated portable chlorophyll fluorometer (and LI-6400-40 leaf chamber fluorometer, Li-Cor, Inc.) following the methods of Betzelberger et al. (2010). In the field, four systems were used, one for each of the experimental blocks, which consisted of one ambient and one elevated [CO₂] treatment. Two systems were first used in ambient plots, and the other two were used first in the elevated [CO₂] plots, each with the same starting time to avoid confounding treatment with time of day. Two to three plants of each cultivar were measured in each plot. Gas exchange was measured at the treatment growth [CO₂] (i.e., 380 ppm for ambient and 550 ppm for elevated [CO₂] treatment plots), ambient air temperature and incident PPFD. Leaf photosynthesis (A), gs, and intercellular [CO₂] (ci) were calculated using the equations of von Caemmerer & Farquhar (1981). Instantaneous water use efficiency was calculated as A/gs.

**Statistical Analysis**

The 18 genotype experiment was analyzed with a randomized complete block split-plot mixed model analysis of variance (Proc MIXED, SAS 9.1, SAS Institute, Cary, NC). [CO₂], year and genotype were fixed effects in the model, and block and block interaction terms were random effects. Correlation analysis was used to test the association between seed yield [CO₂] response ratio (seed yield in elevated [CO₂]/seed yield in ambient [CO₂]) and total above-ground biomass, partitioning coefficient, and height measured at ambient [CO₂] as well as the responses of those variables to elevated [CO₂]. The mean CO₂ response across 2004 and 2005 of each of the 18 genotypes was used for correlation analyses (Proc CORR, SAS 9.1).

The effects of elevated [CO₂] on the nine cultivars that were planted in each of the years 2004 – 2007 was analyzed with a randomized complete block split-plot mixed model analysis of variance (Proc MIXED, SAS 9.1). In all tests, [CO₂], year and genotype were fixed effects in the model, and block and block interaction terms were random effects. In 2008, photosynthetic parameters were analyzed with a randomized complete block split-plot mixed model analysis of variance, with treatment and genotype as fixed effects and block and block interaction terms as random effects (Proc MIXED, SAS 9.1). The two dates on which gas exchange measurements were made were analyzed independently.
Six of the nine genotypes were also grown in 2008. In order to explore year-to-year variation in weather conditions associated with variation in seed yield response to elevated [CO₂] in these six genotypes, correlation analysis of seed yield response to elevated [CO₂] and mean precipitation-potential evaporation (P-PET) in June, July and August was done (Proc CORR, SAS 9.1). An Illinois Climate Network station located approximately 2 km from SoyFACE measured air temperature, incident photosynthetic photon flux density and precipitation throughout the growing seasons (http://www.isws.illinois.edu/atmos/statecli/cuweather/). Daily total potential evaporation data for Champaign-Urbana, IL from 2004-2008 was downloaded from the Illinois Climate Network monitoring program (http://www.isws.illinois.edu/warm/cdflist.asp?typ=a). Potential evapotranspiration data were merged with the precipitation data in order to calculate daily values of P-PET, which is the difference between actual precipitation and potential evapotranspiration, providing a measure of dryness.

Results

CO₂ Response of 18 Soybean Genotypes

Growth at elevated [CO₂] significantly stimulated soybean yields by 8% averaged across 18 different genotypes grown in both 2004 and 2005 (p < 0.0001; Fig. 3.1a). Elevated [CO₂] also stimulated above-ground biomass production (p < 0.0001; Fig. 3.1b), but significantly decreased partitioning of energy to seeds (p < 0.0001; Fig. 3.1c). The effects of elevated [CO₂] on seed yield, above-ground biomass and partitioning coefficient were different in 2004 versus 2005 (significant year x [CO₂] interactions; Table 3.4). In 2004, the average seed yield increase across all 18 genotypes was 20%, but in 2005, there was no difference in yields at ambient and elevated [CO₂] averaged across the 18 genotypes (average yields of 3561 kg ha⁻¹ in ambient [CO₂], 3544 kg ha⁻¹ in elevated [CO₂]). Similarly, the effect of elevated [CO₂] on above-ground biomass varied between 2004 and 2005 (year x [CO₂] interaction, p < 0.0001), with an average increase in biomass at elevated [CO₂] of 32% in 2004, but only 13% in 2005.

Four Year Analysis of the CO₂ Response of 9 Soybean Genotypes

For the nine genotypes grown over the 4 years 2004-2007 (Table 3.2), there was a significant stimulation of yield by elevated [CO₂] averaging 12% across genotypes (Tables 3.5, 3.6). There
was significant variation in the yield response among genotypes, ranging from no significant stimulation in yield in Clark, HS93-4118 and NE3399 to a 24% stimulation in yield in Loda (Table 3.6). Seed yield varied significantly among years, as did the magnitude of the seed yield response to elevated [CO₂] (i.e., significant year x CO₂ interaction; Table 3.5). When the seed yield response to elevated [CO₂] of the nine genotypes was ranked from the most to least responsive, Loda was the most responsive in 3 of the 4 years, and Clark and HS93-4118 were consistently the least responsive (Table 3.7).

Other yield parameters including time to reproductive maturity, plant height, lodging and harvest index were affected significantly by elevated [CO₂] (Table 3.5). On average across nine genotypes and four growing seasons, time to maturity was delayed by 3 days, soybeans were 11 cm taller, experienced more lodging, and the proportion of biomass partitioned to seed declined from 0.55 at ambient [CO₂] to 0.50 in elevated [CO₂] (Table 3.6). There was significant variation among genotypes in all of these traits, but only seed yield and time to maturity also showed a significant [CO₂] x genotype interaction (Table 3.5). Individual seed weight was not significantly affected by growth at elevated [CO₂] (Tables 3.5, 3.6), showing that yield increase resulted from more fertilized ovules and in turn seeds, rather than from larger seeds.

**Correlations Between Seed Yield Response to Elevated [CO₂], Yield Components, and Weather Data**

The mean genotypic responses of each of the 18 genotypes exposed to elevated [CO₂] in 2004 and 2005 was used to investigate how seed yield response to elevated [CO₂] correlated with different traits affecting yield. Total aboveground biomass at ambient [CO₂] was not correlated with the magnitude of the yield response to elevated [CO₂] across genotypes (Fig. 3.2a), but the yield response to elevated [CO₂] was positively and significantly correlated with the CO₂ response of above-ground biomass (Fig. 3.2b). In other words, genotypes that showed the greatest biomass response to elevated [CO₂] also showed the greatest seed yield response. Plant height measured at ambient [CO₂] was negatively correlated with the seed yield CO₂ response (Fig. 3.2c) and there was a significant positive correlation between the response of plant height to elevated [CO₂] and the seed yield [CO₂] response (Fig. 3.2d). There was a positive correlation between genotypes with high partitioning coefficients and their yield response to elevated [CO₂] (Fig. 3.2e) as well as a positive correlation between the change in partitioning coefficient at
elevated [CO₂] and the relative yield response (Fig. 3.2f). Thus, genotypes with greater partitioning of energy to seeds and genotypes that did not show as great a reduction in partitioning coefficient at elevated [CO₂] showed the greatest yield response to elevated [CO₂]. These yield component traits and the correlations among them are not independent. The change in seed mass at elevated [CO₂] was significantly and positively correlated with both biomass change at elevated [CO₂], and significantly and negatively correlated with the partitioning coefficient response to elevated [CO₂] (Table 3.3).

The magnitude of the seed yield response to elevated [CO₂] varied significantly among years (Tables 3.4, 3.5). Therefore, we investigated how variation in weather conditions impacted the mean yield response of 6 genotypes grown at SoyFACE throughout 2004-2008. In those years, mean temperature in June, July and August varied from 20.7 to 23.3 °C and precipitation from 209 to 285 mm (Table 3.2). Across years, temperature and precipitation were negatively correlated, i.e., cooler years tended to be wetter years. P-PET is a measure of soil drying, which is driven by both temperature and precipitation. Across five years of experimentation, there was a weak, but significant positive correlation between P-PET and yield response to elevated [CO₂]. That is, the response of yield to elevated [CO₂] tended to be greater in cooler, wetter years (Fig. 3.3).

**Gas Exchange Responses to Elevated [CO₂]**

In order to determine if variation in yield response to elevated [CO₂] was correlated with photosynthetic response to elevated [CO₂], midday gas exchange measurements were made in 6 genotypes in 2008. On 14 July 2008, when plants were in the V4 growth stage, rates of photosynthesis increased by 26% on average at elevated [CO₂] (Fig. 3.4a), gs decreased by 24% (Fig. 3.4b) and A/gs increased by 68% (Fig. 3.4c). On 31 July 2008, when plants were in R1 growth stage, A was stimulated by 17% on average across genotypes, gs decreased by 24% and A/gs increased by 41% (Fig. 3.5). The response of A, gs or A/gs was not significantly correlated with the yield response to elevated [CO₂] in the six cultivars measured in 2008 (Fig 3.6).

**Discussion**

Significant variation in soybean yield response to elevated [CO₂] is a pre-requisite for breeding to maximize CO₂ response. This study demonstrated that soybean genotypes significantly vary
in their responses of seed yield to elevated [CO₂] from no significant change to an increase in seed yield of more than 20% when measured in 2004 and 2005 (Fig. 3.1a). Despite a universal decrease in the amount of biomass and energy that was partitioned to seed under elevated [CO₂] (Fig. 3.1c), soybean genotypes with the greatest seed yield response to elevated [CO₂] also had large increases in total biomass production at elevated [CO₂] (Fig. 3.1b). Another important finding was that in the nine genotypes used consecutively in each of four years, there was consistency in their response. Clark and HS93-4118 did not increase yield in elevated [CO₂], while Loda was the most responsive to elevated [CO₂] in 3 out of 4 years. Three of the six cultivars that showed a significant seed yield and biomass response to elevated CO₂ (Table 3.6); Loda, Pana and Dwight share Jack as a parent (Table 3.2). While these findings do not demonstrate conclusively that [CO₂] response is genetically controlled, which is critical if there is to be any success in future efforts to breed for CO₂ response, it is encouraging and the first evidence that consistent variation between genotypes can be found under open-air field conditions. One of the consistently unresponsive genotypes examined was Clark, which is an older variety released in 1952 (Table 3.2). It had low yields in both ambient and elevated [CO₂] (Table 3.6). However, both HS93-4118 and Loda were released in 2000 and had similar yields at ambient [CO₂], but very different responses to elevated [CO₂]. As modern, high yielding genotypes, this appears an important resource for identifying the genetic basis of [CO₂] responsiveness of soybean yield.

Soybean yields in the U.S. have quadrupled from the 1920s to today and continue to show a linear trend in yield improvement of 22.2 kg ha⁻¹ yr⁻¹ (Ainsworth et al. 2012). Advances in soybean genetics and the release of new and improved cultivars, improvements in farming technology and management practices, as well as historical increases in atmospheric [CO₂] have contributed to these gains (Specht et al. 1999; McGrath & Lobell 2011; Rowntree et al. 2012). Despite these successes, the current rate of yield improvement in soybean is insufficient to meet the anticipated demand from a growing and more affluent global population (Ray et al. 2013). Thus, new approaches are desperately needed if supply is going to meet accelerating demand. It is also important to consider that while average soybean yields around the globe are increasing, regionally there are locations where yields are decreasing (Ray et al. 2012). Increasing atmospheric [CO₂] and other greenhouse gases are increasing global temperatures and crop demand for moisture, trends that are making it more difficult to maintain, much less improve
upon historical rates of gain (Lobell & Gourdji 2012). Previous studies have suggested that there is very little evidence that breeders have inadvertently selected for increased CO₂ responsiveness, and indeed a number of studies have suggested the opposite, that older genotypes are more responsive to elevated [CO₂] than modern genotypes (reviewed by Ainsworth et al. 2008; Leakey & Lau 2012; Ziska et al. 2012). While the present experiments were not designed to test if [CO₂] response was correlated with genotype year of release, there was significant variation among soybean genotypes in their response to elevated [CO₂].

Variation in the yield response to elevated [CO₂] across a small number of genotypes of soybean has been demonstrated previously under greenhouse conditions (Ziska et al. 1998; 2001; Ziska & Bunce 2000). These studies were critical in demonstrating that there is genotypic variation in the response of soybean elevated [CO₂]. The present study takes the next important step, showing that genotypic variation in soybean response to elevated [CO₂] can also be demonstrated in a typical production environment in the open air repeatedly between years.

Three of the genotypes investigated in this study were previously tested for yield response to elevated [CO₂] in glasshouse conditions. Clark increased seed yield by 28%, Spencer by 45%, and Williams by 40% when grown at elevated [CO₂] (Ziska et al. 1998; 2001). When these genotypes were grown in the field at elevated [CO₂] in this study, there was no significant increase in yield in any of the genotypes (Fig. 3.1), with Clark consistently being the least responsive of all genotypes to elevated [CO₂] (Table 3.7). Clark was also the oldest genotype and perhaps not adapted to the contemporary row spacing used in these experiments. In the present study, consistent responses of diverse soybean genotypes to 4 years of exposure to elevated [CO₂] included increased height, increased lodging and decreased harvest index, as well as increased seed yield. These are traits that may not be apparent in soybean plants grown in isolation, under lower light levels. Another notable difference is that yield gains in the greenhouse experiments were in part attributed to increased branching at elevated [CO₂] (Ziska et al. 1998; 2001), which was not observed in the field, and may be the effect of very different densities in pot experiments versus production field conditions.

This study also showed that the magnitude of the seed yield response to elevated [CO₂] varied among the different years of study. Yield stimulation at elevated [CO₂] tended to be greater in cooler and wetter years, leading to a significant positive correlation between P-PET and the relative yield response to elevated [CO₂] (Fig. 3.3). P-PET is driven by both temperature
and precipitation, and less negative values of P-PET represent more favorable soil moisture conditions or less drought stress. The observation that yield response to elevated [CO$_2$] in soybean was generally greater in wetter years is perhaps a surprising result as generally reduced transpiration of crops at elevated [CO$_2$] conserves soil moisture and therefore it is expected that yield stimulation of C$_3$ crops at elevated [CO$_2$] is greater in times and places of drought. In support of that expectation, a global meta-analysis of C$_3$ crop yield data found greater responsiveness of C$_3$ seed yield under lower precipitation conditions, but where P-PET was not known (Bishop et al. 2014). However, that meta-analysis included all C$_3$ crop species grown at elevated [CO$_2$] in field experiments and results at an individual field site may be very different. Also, soybean exposed to dry soils and elevated [CO$_2$] at SoyFACE produced more nodules in shallow, drier soil layers, which negatively impacted N content and potentially productivity (Gray et al. 2013).

The effects of elevated [CO$_2$] on soybean gene expression, physiology, phenology and yield have been studied extensively at SoyFACE (Rogers et al. 2004; Morgan et al. 2005; Ainsworth et al. 2006; Bernacchi et al. 2006; Castro et al. 2009; Leakey et al. 2009b), but there is still very little understanding of which traits would best predict maximum yield response in different soybean genotypes or in other important crops (Leakey & Lau, 2012; Ziska et al. 2012). Across our panel of 18 soybean genotypes, height was negatively correlated with the magnitude of the yield response (Fig. 3.2c), but the relative response of the trait to elevated [CO$_2$] was positively correlated with the seed yield increase (Fig. 3.2d). Previous work at SoyFACE with two different genotypes showed that increased height at elevated [CO$_2$] occurred at the end of the growing season when additional nodes were added to the plant at elevated [CO$_2$] (Morgan et al. 2005). The increased node number then corresponded to increased pod number per plant and seed yield. Therefore, it is possible that in the 18 genotype panel tested in this study, increased height at elevated [CO$_2$] was also associated with more nodes, and therefore more pods per plant and greater seed yield. There was no statistically significant evidence for a photosynthetic or stomatal basis for the variation in yield response across six genotypes; however, with only six genotypes, there was little power to make strong conclusions.

Our results suggest that an important trait to be targeted in maximizing soybean [CO$_2$] response in the future is the partitioning coefficient, also known as harvest index. The partitioning coefficient decreased on average by 11% across all genotypes, with reductions
ranging from 18% for Flyer to 5% for Dwight (Fig. 3.1c). This consistent decrease indicates sink limitation, i.e., under conditions that generate additional photosynthate, capacity to form additional seed becomes limiting. There was also a positive correlation between changes in partitioning coefficient and yield at elevated [CO₂] (Fig. 3.2f), such that lines with the smallest reductions in partitioning coefficient showed the greatest stimulation in yield. Therefore selection of germplasm that can maintain a high partitioning coefficient under conditions conducive to high productivity will be important in maximizing response to rising [CO₂] in soybean as well as other C₃ crops (Aranjuelo et al. 2013; Hasegawa et al. 2013).

In conclusion, this study demonstrated under fully open-air field conditions that there is significant genetic variation in soybean response to elevated [CO₂], and therefore the potential to breed soybean to an elevated [CO₂] environment. Capitalizing on this genetic variation, along with using on-farm adaptation strategies could help mitigate the expected negative impacts of climate change and potentially improve crop yields in the future. Further studies are needed to test the heritability of [CO₂] response in soybean and in other major crops, as well as to address the methodological challenges of selecting for [CO₂]-responsive germplasm (Ziska et al. 2012).
**Table 3.1.** Meteorological and planting data from 2004 to 2008 at the SoyFACE experimental facility. Average daytime temperature (°C) shows the mean for June, July and August, total precipitation is the sum of all precipitation for June, July and August.

<table>
<thead>
<tr>
<th>Year</th>
<th>Mean temperature (°C)</th>
<th>Total precipitation (mm)</th>
<th>Planting Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>20.7</td>
<td>285</td>
<td>28 May</td>
</tr>
<tr>
<td>2005</td>
<td>23.3</td>
<td>234</td>
<td>25 May</td>
</tr>
<tr>
<td>2006</td>
<td>22.5</td>
<td>281</td>
<td>25 May</td>
</tr>
<tr>
<td>2007</td>
<td>22.8</td>
<td>222</td>
<td>23 May</td>
</tr>
<tr>
<td>2008</td>
<td>22.2</td>
<td>209*</td>
<td>16 June</td>
</tr>
</tbody>
</table>

*measured from planting date, June 16, 2008*
Table 3.2. List and description of soybean genotypes used in this study. *Indicates the genotype was replicated from 2004-2007. †Indicates the genotypes was grown in 2008.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Year of Release</th>
<th>Maturity Group</th>
<th>Parent1</th>
<th>Parent2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3127*</td>
<td>1977</td>
<td></td>
<td>Williams</td>
<td>Essex</td>
</tr>
<tr>
<td>Clark*</td>
<td>1952</td>
<td>IV</td>
<td>Lincoln (2)</td>
<td>Richland</td>
</tr>
<tr>
<td>Dwight*†</td>
<td>1997</td>
<td>II</td>
<td>Jack</td>
<td>A86-303014</td>
</tr>
<tr>
<td>HS93-4118**†</td>
<td>2000</td>
<td>II</td>
<td>IA 2007</td>
<td>DSR 304</td>
</tr>
<tr>
<td>IA 3010*†</td>
<td>1998</td>
<td>III</td>
<td>J285</td>
<td>S29-39</td>
</tr>
<tr>
<td>LN97-15076*†</td>
<td>2003</td>
<td>IV</td>
<td>Macon</td>
<td>Stressland</td>
</tr>
<tr>
<td>Loda*†</td>
<td>2000</td>
<td>II</td>
<td>Jack</td>
<td>IA3003</td>
</tr>
<tr>
<td>NE3399*</td>
<td>1999</td>
<td>III</td>
<td>Holt</td>
<td>Dairyland DSR304</td>
</tr>
<tr>
<td>Pana*†</td>
<td>1997</td>
<td>III</td>
<td>Jack</td>
<td>Asgrow A3205</td>
</tr>
<tr>
<td>Corsoy 79</td>
<td>1988</td>
<td>II</td>
<td>Corsoy (6)</td>
<td>Lee 68</td>
</tr>
<tr>
<td>DSR 304</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flyer</td>
<td>1990</td>
<td>IV</td>
<td>A3127 (4)</td>
<td>L24</td>
</tr>
<tr>
<td>Holt</td>
<td>1992</td>
<td>IV</td>
<td>Sherman</td>
<td>Harper</td>
</tr>
<tr>
<td>IA 2052</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jack</td>
<td>1990</td>
<td>II</td>
<td>Fayette</td>
<td>Hardin</td>
</tr>
<tr>
<td>LG00-6313</td>
<td></td>
<td>IV</td>
<td>Jin Dou 33</td>
<td>Fen dou 31</td>
</tr>
<tr>
<td>Spencer</td>
<td>1988</td>
<td>IV</td>
<td>A75-305022</td>
<td>Century</td>
</tr>
<tr>
<td>Williams</td>
<td>1972</td>
<td>III</td>
<td>Wayne</td>
<td>L57-0034</td>
</tr>
</tbody>
</table>
Table 3.3. Correlation between responses of different agronomic traits to elevated [CO₂]. Statistically significant (p < 0.05) correlations are bolded, marginally significant (p < 0.10) correlations are italicized.

<table>
<thead>
<tr>
<th></th>
<th>Time to Maturity</th>
<th>Height</th>
<th>100 Seed Weight</th>
<th>Aboveground Biomass</th>
<th>Partitioning Coefficient</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to Maturity</td>
<td>$r = 0.44$</td>
<td>$r = 0.57$</td>
<td>$r = 0.19$</td>
<td>$r = 0.07$</td>
<td>$r = 0.17$</td>
<td></td>
</tr>
<tr>
<td>p = 0.07</td>
<td>$p = 0.01$</td>
<td>$p = 0.46$</td>
<td>$p = 0.12$</td>
<td>$p = 0.64$</td>
<td>$p = 0.50$</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>$r = 0.52$</td>
<td>$r = 0.61$</td>
<td>$r = 0.007$</td>
<td>$p = 0.04$</td>
<td>$r = 0.48$</td>
<td></td>
</tr>
<tr>
<td>p = 0.03</td>
<td>$p = 0.01$</td>
<td>$p = 0.001$</td>
<td>$p &lt; 0.001$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed Weight</td>
<td>$r = 0.57$</td>
<td>$r = 0.59$</td>
<td>$p = 0.01$</td>
<td>$r = 0.70$</td>
<td>$p = 0.001$</td>
<td></td>
</tr>
<tr>
<td>Aboveground Biomass</td>
<td></td>
<td>$r = 0.19$</td>
<td>$p = 0.44$</td>
<td>$p &lt; 0.001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partitioning Coefficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$r = 0.70$</td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$p = 0.001$</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4. Analysis of variance of the response of 18 soybean genotypes exposed to ambient and elevated [CO₂] in 2004 and 2005. Results from the mixed model analysis of variance (F test) and statistical significance (p) for each source of variation are shown. Significant effects are shown in bold.

<table>
<thead>
<tr>
<th></th>
<th>Seed Yield</th>
<th>Aboveground Biomass</th>
<th>Partitioning Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (G)</td>
<td>12.0, &lt;0.001</td>
<td>6.51, &lt;0.001</td>
<td>26.4, &lt;0.001</td>
</tr>
<tr>
<td>CO₂</td>
<td>15.4, &lt;0.001</td>
<td>88.9, &lt;0.001</td>
<td>145.5, &lt;0.001</td>
</tr>
<tr>
<td>G x CO₂</td>
<td>0.78, 0.720</td>
<td>0.61, 0.883</td>
<td>1.39, 0.141</td>
</tr>
<tr>
<td>Year</td>
<td>0.59, 0.443</td>
<td>0.13, 0.714</td>
<td>1.16, 0.283</td>
</tr>
<tr>
<td>G x Year</td>
<td>1.25, 0.231</td>
<td>0.81, 0.682</td>
<td>3.67, &lt;0.001</td>
</tr>
<tr>
<td>CO₂ x Year</td>
<td>17.1, &lt;0.001</td>
<td>12.64, &lt;0.001</td>
<td>7.52, 0.007</td>
</tr>
<tr>
<td>G x CO₂ x Year</td>
<td>0.55, 0.924</td>
<td>0.44, 0.973</td>
<td>0.76, 0.739</td>
</tr>
</tbody>
</table>
**Table 3.5.** Analysis of variance of the response characteristics of 9 soybean genotypes exposed to ambient and elevated [CO₂] from 2004-2007. Results from the mixed model analysis of variance (F test) and statistical significance (P) for each source of variation are shown. Significant effects are shown in bold.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Seed Yield</th>
<th>Aboveground Biomass</th>
<th>100 Seed Weight</th>
<th>Partitioning Coefficient</th>
<th>Height</th>
<th>Lodging</th>
<th>Time to Maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (G)</td>
<td>37.9, &lt;0.001</td>
<td>5.37, &lt;0.001</td>
<td>21.3, &lt;0.001</td>
<td>62.5, &lt;0.001</td>
<td>172.4, &lt;0.001</td>
<td>37.8, &lt;0.001</td>
<td>241.0, &lt;0.001</td>
</tr>
<tr>
<td>CO₂</td>
<td>33.9, &lt;0.001</td>
<td>45.3, &lt;0.001</td>
<td>2.59, 0.109</td>
<td>119.0, &lt;0.001</td>
<td>191.8, &lt;0.001</td>
<td>71.3, &lt;0.001</td>
<td>215.2, &lt;0.001</td>
</tr>
<tr>
<td>G x CO₂</td>
<td>2.74, 0.007</td>
<td>1.06, 0.397</td>
<td>0.85, 0.560</td>
<td>1.56, 0.141</td>
<td>1.58, 0.133</td>
<td>1.21, 0.294</td>
<td>5.59, &lt;0.001</td>
</tr>
<tr>
<td>Year</td>
<td>42.8, &lt;0.001</td>
<td>0.83, 0.364</td>
<td>5.68, 0.004</td>
<td>1.29, 0.278</td>
<td>193.4, &lt;0.001</td>
<td>19.0, &lt;0.001</td>
<td>425.6, &lt;0.001</td>
</tr>
<tr>
<td>G x Year</td>
<td>2.25, 0.001</td>
<td>0.58, 0.795</td>
<td>3.42, &lt;0.001</td>
<td>3.80, &lt;0.001</td>
<td>1.69, 0.027</td>
<td>1.89, 0.009</td>
<td>4.96, &lt;0.001</td>
</tr>
<tr>
<td>CO₂ x Year</td>
<td>9.97, &lt;0.001</td>
<td>9.50, 0.003</td>
<td>3.60, 0.029</td>
<td>5.59, 0.005</td>
<td>0.21, 0.893</td>
<td>6.10, 0.001</td>
<td>6.19, 0.001</td>
</tr>
<tr>
<td>G x CO₂ x Year</td>
<td>0.72, 0.824</td>
<td>0.14, 0.997</td>
<td>0.78, 0.707</td>
<td>1.06, 0.402</td>
<td>0.34, 0.998</td>
<td>1.11, 0.329</td>
<td>1.83, 0.013</td>
</tr>
</tbody>
</table>
Table 3.6. Yield and yield parameters of soybean genotypes exposed to ambient (Amb) and elevated [CO2] (Ele) from 2004-2007 (mean ± 1 std err). Yield (kg ha⁻¹), aboveground biomass (g), mass of 100 seeds (g), partitioning coefficient, height (cm), lodging score (1-10), and time to reach reproductive stage 8 (d). Significant differences (p<0.05) between ambient and elevated [CO2] within a genotype are shown in bold.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>[CO2]</th>
<th>Yield</th>
<th>Aboveground Biomass</th>
<th>Mass of 100 seeds</th>
<th>Partitioning Coefficient</th>
<th>Height</th>
<th>Lodging</th>
<th>Time to Maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>Amb</td>
<td>3255 ± 61</td>
<td>1112 ± 18.6</td>
<td>15.8 ± 0.15</td>
<td>0.55 ± 0.005</td>
<td>107 ± 1.5</td>
<td>2.9 ± 0.10</td>
<td>117 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>3631 ± 87</td>
<td>1350 ± 34.8</td>
<td>16.0 ± 0.15</td>
<td>0.50 ± 0.006</td>
<td>118 ± 1.6</td>
<td>3.9 ± 0.16</td>
<td>120 ± 0.5</td>
</tr>
<tr>
<td>A3127</td>
<td>Amb</td>
<td>3029 ± 145</td>
<td>1059 ± 54.1</td>
<td>14.8 ± 0.32</td>
<td>0.53 ± 0.01</td>
<td>101 ± 3.0</td>
<td>1.9 ± 0.17</td>
<td>116 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>3450 ± 256</td>
<td>1284 ± 110.3</td>
<td>15.0 ± 0.32</td>
<td>0.49 ± 0.02</td>
<td>108 ± 3.1</td>
<td>2.8 ± 0.28</td>
<td>117 ± 1.1</td>
</tr>
<tr>
<td>Clark</td>
<td>Amb</td>
<td>2159 ± 165</td>
<td>906 ± 59.6</td>
<td>16.3 ± 0.59</td>
<td>0.47 ± 0.02</td>
<td>121 ± 2.6</td>
<td>4.7 ± 0.27</td>
<td>121 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>1978 ± 142</td>
<td>986 ± 105.0</td>
<td>15.9 ± 0.38</td>
<td>0.39 ± 0.01</td>
<td>131 ± 2.7</td>
<td>5.9 ± 0.51</td>
<td>124 ± 1.4</td>
</tr>
<tr>
<td>Dwight</td>
<td>Amb</td>
<td>3747 ± 140</td>
<td>1155 ± 37.9</td>
<td>14.0 ± 0.21</td>
<td>0.59 ± 0.21</td>
<td>94 ± 3.3</td>
<td>2.7 ± 0.18</td>
<td>109 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>4465 ± 149</td>
<td>1409 ± 55.2</td>
<td>14.9 ± 0.27</td>
<td>0.57 ± 0.008</td>
<td>104 ± 3.4</td>
<td>3.2 ± 0.37</td>
<td>114 ± 0.9</td>
</tr>
<tr>
<td>HS93-4118</td>
<td>Amb</td>
<td>3489 ± 138</td>
<td>1213 ± 43.4</td>
<td>16.3 ± 0.29</td>
<td>0.54 ± 0.008</td>
<td>109 ± 2.5</td>
<td>3.1 ± 0.20</td>
<td>122 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>3479 ± 246</td>
<td>1281 ± 138.8</td>
<td>16.2 ± 0.39</td>
<td>0.48 ± 0.01</td>
<td>118 ± 3.6</td>
<td>4.5 ± 0.42</td>
<td>124 ± 1.2</td>
</tr>
<tr>
<td>IA-3010</td>
<td>Amb</td>
<td>3462 ± 112</td>
<td>1069 ± 34.0</td>
<td>16.1 ± 0.31</td>
<td>0.59 ± 0.01</td>
<td>89 ± 2.2</td>
<td>1.7 ± 0.12</td>
<td>115 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>3877 ± 192</td>
<td>1371 ± 74.4</td>
<td>16.4 ± 0.33</td>
<td>0.55 ± 0.007</td>
<td>102 ± 2.3</td>
<td>2.2 ± 0.16</td>
<td>117 ± 1.4</td>
</tr>
<tr>
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<td>Amb</td>
<td>3089 ± 102</td>
<td>1110 ± 37.3</td>
<td>17.5 ± 0.28</td>
<td>0.52 ± 0.007</td>
<td>116 ± 3.2</td>
<td>3.2 ± 0.29</td>
<td>124 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Ele</td>
<td>3480 ± 138</td>
<td>1379 ± 78.8</td>
<td>17.4 ± 0.49</td>
<td>0.47 ± 0.007</td>
<td>126 ± 2.8</td>
<td>4.1 ± 0.45</td>
<td>126 ± 1.4</td>
</tr>
<tr>
<td>Loda</td>
<td>Amb</td>
<td>3625 ± 171</td>
<td>1114 ± 53.9</td>
<td>16.8 ± 0.23</td>
<td>0.62 ± 0.007</td>
<td>88 ± 2.3</td>
<td>2.4 ± 0.16</td>
<td>109 ± 0.8</td>
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<tr>
<td></td>
<td>Ele</td>
<td>4494 ± 204</td>
<td>1394 ± 71.0</td>
<td>17.5 ± 0.38</td>
<td>0.58 ± 0.008</td>
<td>101 ± 2.5</td>
<td>3.5 ± 0.33</td>
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</tr>
<tr>
<td>NE3399</td>
<td>Amb</td>
<td>3334 ± 220</td>
<td>1226 ± 60.0</td>
<td>15.6 ± 0.39</td>
<td>0.56 ± 0.01</td>
<td>109 ± 3.1</td>
<td>2.4 ± 0.15</td>
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</tr>
<tr>
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<td>1474 ± 101.0</td>
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<td>0.52 ± 0.008</td>
<td>120 ± 3.3</td>
<td>3.4 ± 0.34</td>
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</tr>
<tr>
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<td>1157 ± 45.2</td>
<td>14.6 ± 0.34</td>
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<tr>
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<td>0.48 ± 0.008</td>
<td>151 ± 3.9</td>
<td>5.8 ± 0.33</td>
<td>125 ± 0.9</td>
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Table 3.7. Ranks of genotypes yield response to elevated [CO$_2$] in each year of the study, where 1 is the rank of the most responsive genotype and 9 is the least responsive.

<table>
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<th>2006</th>
<th>2007</th>
<th>Average Rank</th>
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<td>9</td>
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<td>5</td>
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<tr>
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<td>7</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Loda</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
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<td>7</td>
<td>3</td>
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</tr>
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<td>1</td>
<td>8</td>
<td>3</td>
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</table>
Figure 3.1. Genotypic variation in (a) soybean seed yield, (b) above-ground biomass and (c) partitioning coefficient response to elevated [CO₂]. CO₂ response values are the mean value of each trait in elevated [CO₂]/ambient [CO₂]. Bars show the mean yield +/- 1 standard error in 18 genotypes grown at the SoyFACE facility in 2004 and 2005. Bars with asterisk(s) indicate significant effects of [CO₂] for each genotype tested with linear contrasts. Significance level is based on the difference between the CO₂ treatments, although the data presented are the ratio (FACE/Ambient). (*) p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 3.2. Correlation between seed yield CO$_2$ response and (a) the above-ground biomass measured at ambient [CO$_2$], (b) the above-ground biomass response to elevated [CO$_2$], (c) seed yield CO$_2$ response and height measured in ambient [CO$_2$], (d) seed yield CO$_2$ response and the response of height to elevated [CO$_2$], (e) partitioning coefficient measured at ambient [CO$_2$], and (f) the partitioning coefficient response to elevated [CO$_2$]. Each data point represents the mean seed yield response to elevated [CO$_2$] for an individual cultivar, averaged for 2004 and 2005.
Figure 3.3. Correlation between seed yield CO$_2$ response of six genotypes and the mean daily precipitation – potential evapotranspiration (P-PET, mm) measured in June, July and August in 2004, 2005, 2006, 2007 and 2008.
Figure 3.4. (a) Midday photosynthetic carbon assimilation rate ($A$), (b) stomatal conductance ($g_s$) and (c) instantaneous water use efficiency ($A/g_s$) measured on 14 July 2008 in 6 soybean genotypes exposed to ambient (white bars) or elevated $[\text{CO}_2]$ (black symbols). $G$, $\text{CO}_2$ and $G \times \text{CO}_2$ indicate statistical significance of genotype, $[\text{CO}_2]$ treatment or the interaction of genotype x $[\text{CO}_2]$ treatment.
Figure 3.5. (a) Midday photosynthetic carbon assimilation rate ($A$), (b) stomatal conductance ($g_s$) and (c) instantaneous water use efficiency ($A/g_s$) measured on 31 July 2008 in 6 soybean genotypes exposed to ambient (white bars) or elevated [$CO_2$] (black symbols). G, $CO_2$ and G x $CO_2$ indicate statistical significance of genotype, [$CO_2$] treatment or the interaction of genotype x [$CO_2$] treatment.
Figure 3.6. Lack of correlation between (a) seed yield CO\textsubscript{2} response and the response of photosynthesis ($A$) to elevated [CO\textsubscript{2}], (b) seed yield CO\textsubscript{2} response and the response of stomatal conductance ($g_s$) to elevated [CO\textsubscript{2}] and (c) seed yield CO\textsubscript{2} response and the response of instantaneous water use efficiency ($A/g_s$) to elevated [CO\textsubscript{2}].
CHAPTER IV: DOES PHLOEM LOADING STRATEGY IMPACT PHOTOSYNTHETIC RESPONSE TO ELEVATED [CO₂]?

Abstract
Increased atmospheric [CO₂] stimulates photosynthesis of C₃ plants, leading to increased biomass and crop yield. At elevated [CO₂], however, sugars can accumulate in leaf mesophyll cells, resulting in a negative feedback on photosynthetic capacity. This phenomenon has only been tested in a narrow range of species, mostly those which use proton-sucrose symporters to load sucrose into the companion cells of the phloem (apoplastic loading). Other species use passive diffusion (passive loading), in which source leaf mesophyll cells accumulate high concentrations of sucrose which subsequently enters the phloem passively through plasmodesmata. It was hypothesized that species with passive phloem loading would be adapted to high mesophyll sucrose concentrations, and therefore would not show the same degree of down-regulation of photosynthetic capacity at elevated [CO₂] as species with apoplastic loading.

Pea and beet (apoplastic phloem loaders) and strawberry and peony (passive phloem loaders) were grown at ambient (~400 ppm) and elevated (600 ppm) [CO₂] under fully open-air field conditions for two growing seasons. Pea and strawberry both produce fruit and grow as vines while beet and peony both have large belowground storage organs. All species significantly increased diurnal photosynthetic C assimilation across the growing seasons (by 21-51%) and showed very minimal decreases in photosynthetic capacity at elevated [CO₂], as measured by maximum Rubisco activity and maximum electron transport. All species showed large increases in leaf starch but little increase in leaf soluble sugar content at elevated [CO₂]. The analysis did not show differences in the photosynthetic response of apoplastic and passive loading species to elevated [CO₂], although CO₂ response curves revealed a significant difference in the transition point between Rubisco-limited and electron transport-limited photosynthesis in the two types, with passive loading species having much higher (496-522 ppm c₅) transition points than apoplastic loading species (324-334 ppm c₅). Therefore, photosynthesis was Rubisco-limited at both ambient and elevated [CO₂] in passive loading species, while photosynthetic limitation shifted from Rubisco to electron transport in apoplastic loading species. Contrary to our initial hypothesis, the results indicate little effect of phloem loading strategy on [CO₂] response across the species studied and there was little downregulation of photosynthesis in this experiment.
Introduction
Phloem delivers sugars produced in the mesophyll of leaves to the rest of the plant and is essential for plant productivity (Ainsworth & Bush 2011). In order to facilitate mass flow of sugars from source tissues (leaves) to sink tissues, the phloem near mesophyll cells in the leaf must contain high concentrations of sucrose. Plants have evolved different mechanisms of transporting sucrose to the phloem to maintain those high concentrations (Rennie & Turgeon 2009; Turgeon 2010). The three primary strategies are apoplastic loading, passive loading, and polymer trapping. In apoplastic loading species, sucrose diffuses through the mesophyll until it reaches the phloem and is exported into the apoplast by SWEET transporters (Chen 2014). This apoplastic sucrose is then actively imported into the phloem using proton motive force via proton-sucrose symporters (SUT/SUC) (Lalonde et al. 2004; Yadav et al. 2015). In passive loading species, there is symplastic continuity between the mesophyll and phloem and high sucrose levels in both cell types (Rennie & Turgeon 2009; Yadav et al. 2015). This allows sucrose to passively diffuse into the phloem without an active concentrating step (Rennie & Turgeon 2009). In polymer trapping species, sucrose passively diffuses from the mesophyll cells through plasmodesmata into the intermediary cells, where it is turned into raffinose-family oligomers such as raffinose and stachyose (Rennie & Turgeon 2009). The oligomers are too large to diffuse back through plasmodesmata, hence the polymers are trapped in the intermediary cells. They then diffuse through larger plasmodesmata to the phloem sieve elements, contributing to lowering water potential and a concomitant influx of water and pressure potential that drives mass flow of phloem sap (Rennie & Turgeon 2009).

Many methods have been used to identify plant characteristics specific to each phloem loading strategy since their discovery. Anatomical analyses of plasmodesmata between mesophyll and phloem cells have been used to characterize a wide range of species (Gamalei 1989; 1991; Davidson et al. 2011), although species with numerous plasmodesmata do not always use passive loading (Goggin et al. 2001; Rennie & Turgeon 2009). Other early analyses included, for example, tracer studies using C14 sucrose and studies which blocked sucrose transporters using p-chloro-mercuribenzenesulfonic acid (PCMBS) (Giaquinta 1976; Wimmers & Turgeon 1991). Some more recent surveys of phloem loading strategy have used autoradiography in combination with previous methods (Rennie & Turgeon 2009; Fu et al. 2011). Although species have predominantly been characterized as using one phloem loading
strategy or another, some have been shown to exhibit multiple strategies (Voitsekhovskaja et al. 2009) or can use alternative strategies under certain environmental conditions (Goggin et al. 2001; Srivastava et al. 2009a; Gil et al. 2011).

Many studies have demonstrated the link between photosynthetic capacity and the demand of photosynthate from sink tissue (Plaut et al. 1987; Krapp & Stitt 1995; Paul & Foyer 2001). Amiard et al. (2005) analyzed how phloem loading strategy could impact flexibility in photosynthetic capacity by comparing apoplastic loading and polymer trapping species in their ability to acclimate to a higher light environment. Apoplastic loading species were able to increase their photosynthetic capacity and maintain a low sucrose content in the leaves after transfer from low to high light, but polymer trapping species could not fully increase their photosynthetic capacity and had a buildup of starch in the leaf (Amiard et al. 2005; Adams et al. 2007). Their findings supported the hypothesis that polymer trapping species would be dependent on anatomy to load phloem and so fully developed leaves would be less able to acclimate to a new light environment than apoplastic loading species. The association between phloem loading and photosynthetic capacity, however, has not yet been demonstrated in long-term experiments manipulating source availability.

Increasing carbon dioxide can serve as a method to increase source availability over a longer duration. Elevated carbon dioxide ([CO₂]) increases plant photosynthesis, but can also lead to a buildup of carbohydrates in the leaves (Moore et al. 1998; Rogers et al. 2004; Ainsworth & Long 2005). These excess carbohydrates can then cause a down-regulation in Rubisco content (Moore et al. 1999), therefore decreasing photosynthetic capacity. Similarly, plants with greater sink strength tend to have greater photosynthetic enhancement at elevated [CO₂] and little to no accumulation of leaf carbohydrates (Ainsworth et al. 2004; Aranjuelo et al. 2013). This suggests a link between sucrose translocation to sink tissue and response to elevated [CO₂]. Trees exhibit less photosynthetic acclimation to elevated [CO₂], often attributed to their greater sink capacity (Ainsworth & Long 2005; Davey et al. 2006; Ainsworth & Rogers 2007). The role of sucrose transport strategy, however, in response to long-term CO₂ enrichment has not been thoroughly studied.

Only one short-term experiment has analyzed the effect of phloem loading strategy on response to elevated [CO₂] (Körner et al. 1995). In this experiment, both apoplastic and symplastic loading species increased their total nonstructural carbohydrates (TNC) at elevated
[CO$_2$] and symplastic loading species had greater TNC than apoplastic loading species at both ambient and elevated [CO$_2$]. Körner et al. (1995), however, did not differentiate the symplastic loading species between passive and polymer trapping species, and the elevated [CO$_2$] treatment was very brief, lasting only six to ten days. Therefore, there was little time for anatomical acclimation to elevated [CO$_2$]. In this study, a longer-term approach was used to test the prediction that phloem loading strategy plays a role in photosynthetic acclimation to elevated [CO$_2$]. This prediction was tested in the field through a two year comparison of two apoplastic loading and two passive loading herbaceous species grown at elevated [CO$_2$].

Materials and Methods

Site Description

This experiment was conducted in a mini FACE system at the SoyFACE facility (www.igb.illinois.edu/soyface) at the University of Illinois at Urbana-Champaign; 40°03’21.3” N, 88°12’3.4” W, 230 m elevation, described previously in Bishop et al. (2015). At the start of the experiment in 2013, six square plots were established with a side length of 3.66 m. Three of these plots were fumigated to a CO$_2$ concentration of 600 ppm, and the other three were left as controls and were not fumigated. The plots were separated by 3.66-7.32 m, as described in Fig. 4.1. [CO$_2$] in the ambient plots showed minimal cross-contamination from the elevated plots (Fig. 4.2).

The CO$_2$ fumigation system was modified from Miglietta et al. (2001b). The first modification was that the plots were squares rather than octagons. The second was that instead of having a separate anemometer and CO$_2$ control program in each elevated plot, one plot (plot 3 in Fig. 4.1) was the “master”, which controlled both its fumigation and the fumigation of the other two plots (plots 1 and 5 in Fig. 4.1). Therefore, the wind speed and [CO$_2$] measured by a combination wind vane and 3-cup anemometer in plot 3 were used to control the CO$_2$ fumigation volume and direction for all three elevated [CO$_2$] plots. The third modification was that only one row of pipe was used per side of the plot, unlike two in the setup of Miglietta et al. (2001b).

CO$_2$ fumigation began on April 29, 2013 and April 21, 2014 and concluded on July 5, 2013 and July 2, 2014. In 2013, ten minute average [CO$_2$]s were within 10% of the target for

5 Fumigation set up by Christopher Montes
68.4% of the time, and within 20% of the target 89.1% of the time (Fig. 4.2a). The season-long mean elevated [CO₂] was 621.3 ppm. The master plot had more accurate fumigation than the other two plots (Fig. 4.2). Approximately 22 days of fumigation data from 2014 were lost due to a computer error, but the seasonal data were similar to 2013. Ten minute averages were within 10% of the target 66.4% of the time, and within 20% of the target 92.1% of the time (Fig 4.2b). The mean elevated [CO₂] was 586.6 ppm in 2014.

Planting

Peony (Paeonia lactiflora) cv Edulis Superba, beet (Beta vulgaris) cv Detroit Dark Red, pea (Pisum sativum) cv Frosty, and strawberry (Fragaria x ananassa) cv Ft Laramie were grown in the field at the SoyFACE experimental site. These species were selected because they had similar growth habits and had been previously characterized by phloem loading type, which few species have been (Rennie & Turgeon 2009; Fu et al. 2011). Beet and peony both have belowground storage organs, while pea and strawberry both produce fruit and are vines. Beet and pea had previously been characterized as apoplastic loading species by Giaquinta (1976) and Wimmers & Turgeon (1991). Peony and strawberry had been characterized as passive loading species by Rennie & Turgeon (2009). Each species occupied one 1.83 m x 1.83 m quadrant (Figure 4.1). All plots were tilled prior to planting except for the peony plots in 2014. Plants were fertilized every one to two weeks using a 12-4-8 NPK fertilizer (LiquaFeed All-Purpose, Miracle-Gro, Marysville, OH, USA). In 2014, the entire area was mulched with Miscanthus x giganteus stover on May 23 to prevent weed growth and evaporation. Weather data was collected from the Champaign Willard Airport (~2.4 km away). Season-long average temperature was 18.5° C in 2013 and 19.3° C in 2014. Season-long precipitation was 337.8 mm in 2013 and 404.4 mm in 2014, with additional irrigation to prevent drought stress.

For the 2013 growing season, peonies were put into pots on November 6, 2012, covered with mulch, and left to overwinter at the field site. The roots were then planted in the ground on April 2, 2013. For the 2014 growing season, peonies were planted directly in the ground on December 4, 2013. Four peonies were planted per plot (Fig. 4.3). Peonies emerged in mid to late April in both 2013 and 2014. Beets and peas were planted as seeds on April 26, 2013 and April 18, 2014, with planting densities as described in Fig. 4.3. Pea plants were grown on stakes. Strawberries were planted as small plants on May 11, 2013 and April 18, 2014 (Fig. 4.3) with a
total of eight plants per plot. Runners were contained to within the strawberry plot and at least 30 cm from the outer edge of each plot. The beet and pea plots were over-seeded so plants were thinned to the desired amount (five beets and ten peas per plot) on May 13 (pea) and May 20 (beet), 2013 and May 14, 2014. In 2013, cages were built around beet plots after damage from wildlife was noticed. In 2014, a 1m fence was built around all plots prior to plant emergence.

Gas Exchange Measurements

Diurnal photosynthesis measurements were taken every 2-3 hours from dawn to dusk on May 29 and June 19, 2013 and May 30 and June 17, 2014. Two portable open infrared gas-exchange systems (LI-6400, Li-Cor Inc, Lincoln, NE, USA) were used with a 2 cm² circular leaf chamber. Measurements were performed on sunlit fully expanded leaves at the top of the canopy, which had developed at elevated [CO₂]. Immediately before a time-point, light and temperature were measured using local weather data and a light meter just above the plant canopy (LI-210, LiCor, Inc). The block was maintained at those conditions throughout that time-point. Leaf photosynthesis (A), stomatal conductance (gs), and intercellular CO₂ concentration (ci) were calculated using the equations from von Caemmerer & Farquhar (1981). The total daily carbon uptake (A') was calculated by integrating under the curve of A measurements. Beet plants were too small to measure in May 2013.

Measurements of A vs ci were taken on two plants per species per plot on May 30 and June 19-20, 2013 and May 31-June 1 and June 21-22, 2014 using four open gas exchange systems (LI-6400, Li-Cor). Measurements were taken on sunlit fully expanded intact leaves, and initiated at growth [CO₂]. [CO₂] in the chamber was then decreased stepwise to 50 ppm before returning to growth [CO₂] and increasing to 1500 ppm. From these response curves, maximum rate of Rubisco carboxylation (Vc,max), maximum rate of electron transport (Jmax), the transition point, and dark respiration (Rd) were determined using the equations of Farquhar et al. (1980) as described by Long & Bernacchi (2003). Although the block temperature was set at 25°C, the actual leaf temperature in the field ranged from 25 to 36°C. To account for this, photosynthetic parameters were estimated at 25°C using the temperature corrections of Bernacchi et al. (2001, 2003). All curves were taken at saturating light (2000 μmol quanta m⁻² s⁻¹) and were performed between dawn (0600) and midday (1300).
Leaf Carbohydrate and Nitrogen Content

Diurnal sugar profiles were analyzed by taking leaf punches (12.5 mm dia) at dawn (0500), midday (1200-1300), dusk (1930), and dawn (0500) the following day on the same day as the June diurnal photosynthesis measurements. Samples were immediately frozen in liquid N. Peonies were not sampled in 2014 due to low emergence in some plots. The samples were analyzed for glucose, fructose, and sucrose content using the methods of Jones et al. (1977). Starch was extracted and digested and content was determined as glucose equivalents using the methods of Hendricks et al. (2003). Two additional leaf discs (12.5 mm dia) were taken to determine specific leaf area and N content at midday on the same day. These discs were dried at 55°C for one week and ground. 2.8-3.2 mg of leaf powder was weighed and placed in tin capsules for elemental analysis. The samples were combusted using chromium oxide as a catalyst and all N and C compounds were reduced to N2 and CO2 for detection by an elemental analyzer (ECS 4010, Costech Analytical Technologies Inc., Valencia, CA, USA). Acetanalide and NIST apple leaves were used as standards.

Leaf Anatomy

Leaf samples for microscopy (5.5 mm dia) were taken on June 25, 2014 and immediately fixed in phosphate buffered 2% glutaraldehyde and 2.5% paraformaldehyde (Karnovsky’s fixative). Microwave fixation was used with the primary fixative and the tissue was then washed in Sorenson’s phosphate buffer with no further additives. Microwave fixation was also used with the secondary 2% osmium tetroxide fixative, followed by the addition of 3% potassium ferricyanide at the end of the osmium incubation. After washing with water, saturated uranyl acetate was added for en-bloc staining.

The tissue was dehydrated in a series of washes with increasing concentrations of ethanol. Acetonitrile was used as the transition fluid between ethanol and the epoxy. Infiltration series was done with an epoxy mixture using the epon substitute Lx112. The resulting blocks were polymerized at 90°C overnight, trimmed and ultrathin sectioned with an ultramicrotome (Reichert-Jung Ultracut E, Reichert Microscope Services, Depew, NY).

Thick sections (0.35-0.45 µm) were taken for light microscopy. Sections were stained with Toluidine Blue and basic fuchsine and analyzed with a light microscope (Leica DM2000,
Leica Microsystems Inc., Wetzlar, Germany). Images were processed using ImageJ version 1.48 (http://rsb.info.nih.gov/ij/). Percent air space was calculated by taking a 250 µm wide section of the leaf image (150 µm for strawberry), avoiding large veins and the epidermis, and then dividing the area of air space by the total area analyzed.

Thin sections were stained with uranyl acetate and lead citrate, and photographed with a transmission electron microscope (H600, Hitachi, Ltd, Tokyo, Japan). Pea cell wall invaginations were analyzed as described in Amiard et al. (2005) using ImageJ.

Statistics
All measured parameters were analyzed using a mixed model analysis of variance (PROC MIXED, SAS 9.3; SAS Institute, Cary, NC, USA), with [CO2] and species as fixed effects. Parameters measured multiple times throughout the day (A, g, carbohydrates) were analyzed with a repeated measures mixed model analysis of variance. All parameters which were measured across multiple dates but not multiple times on each date were analyzed with date as a fixed effect. When necessary, data were transformed to fit the assumptions of ANOVA. A log transformation was used for total soluble sugar and starch content in 2014, the transition point, C:N ratio, and spongy mesophyll thickness. A square root transformation was used for total soluble sugar and starch content in 2013 and stomatal conductance on May 2013. When there was a significant infrared gas analyzer effect for diurnal A or g measurements (p < 0.05), this was included into the model. A point was discarded as an outlier if the residual was more than three times the interquartile range from the rest of the residuals.

Meta-Analysis
A second test for differences in [CO2] response among species with different phloem loading strategies was done using meta-analysis. A list of species which had been previously characterized as passive or apoplastic loading was taken from Rennie & Turgeon (2009) and Fu et al. (2011). Both of these studies used autoradiography in addition to anatomical characterization, which can provide an accurate determination of phloem loading strategy (Rennie & Turgeon 2009). A search for papers which reported measurements of A at ambient and elevated [CO2] in these characterized species was performed using ISI Web of Knowledge (Thomson ISI, Philadelphia, PA, USA). The analysis was limited to trees rooted in the ground,
measurements taken with open gas exchange systems, and measurements taken at saturating light from leaves at the top of the canopy. Herbaceous species were excluded because there were few studies of characterized herbaceous species. The meta-analysis was restricted to the highest N availability and to studies without additional abiotic stress in order to minimize interactive effects. Parameter values for different genotypes, CO₂ treatments, or years were assumed to be independent and were included separately, as in previous meta-analyses (Curtis & Wang 1998; Medlyn et al. 1999). Parameters measured on the same plants throughout one growing season were averaged across the sampling dates. Mean values, standard deviation of the mean, and sample size were taken from tables or extracted from figures using digitizing software (Grafula 3 v.2.10, Wesik SoftHaus, St. Petersburg, Russian Federation). Overall, four passive loading species and three apoplastic loading species were included in this analysis.

A total of 26 primary manuscripts were used for this analysis (Appendix B). All studies had an elevated [CO₂] treatment of 530 to 720 ppm. The natural log of the response ratio (r = response in elevated [CO₂] / response in ambient [CO₂]) was used for all analyses (Hedges et al. 1999; Rosenberg et al. 2000). A weighted analysis was performed following previous methods (Hedges et al. 1999) using the statistical software MetaWin (Rosenberg et al. 2000). For the one study which did not report variances, an average of the variance across the rest of the experiments was used. Since a weighted analysis was used, the total heterogeneity (Qₜ) was partitioned to within (Qₜ) and between (Qₖ) categorical variables (Curtis & Wang 1998). Effects were considered significant when the Qₖ value was below 0.05.

Results

Gas Exchange Responses to Elevated [CO₂]

Across all species and measurement dates, there was a significant increase in diurnal A at elevated [CO₂] (Table 4.1, Fig. 4.4). When measurements were integrated throughout the day to determine daily C gain (A'), the increase at elevated [CO₂] was between 21 and 51% (Table 4.2, Fig. 4.5). There was a highly significant species x [CO₂] effect in A' (Table 4.2), which was largely consistent across dates (i.e., date x [CO₂] and date x species x [CO₂] effects were not significant, Table 4.2). This variation among species did not correspond to differences in phloem loading strategy, since strawberry, a passive loading species, had the greatest stimulations in A', followed closely by beet, an apoplastic loading species. Peony, a passive
loading species, and pea, an apoplastic loading species, tended to have the lowest stimulations in $A'$ (Figs. 4.4, 4.5).

Stomatal conductance decreased slightly at elevated [CO$_2$], primarily during the June measurements (Table 4.1, Fig. 4.6). Although the time x [CO$_2$] interaction was only significant on one of the dates, most of the significant decreases in $g_s$ were observed in the late afternoon (Fig. 4.6).

Averaged across measurement dates and species, $V_{c,max}$ was slightly reduced at elevated [CO$_2$] (by 6%), and there was a marginally significant reduction in $J_{max}$ (Table 4.2, Figs. 4.7, 4.8). However, most within-species contrasts did not reveal significant differences in $V_{c,max}$ or $J_{max}$ on individual days, suggesting that photosynthetic capacity was only modestly affected by elevated [CO$_2$].

The transition point between Rubisco- and electron transport-limited photosynthesis was significantly different between species (Table 4.2, Fig. 4.9) across all dates and CO$_2$ concentrations. This difference was associated with phloem loading strategy: the apoplastic loading species beet and pea had average transition points of 324 and 334 ppm c$_i$ for plants grown at ambient [CO$_2$], and the passive loading species peony and strawberry had average transition points of 522 and 496 ppm c$_i$ (Fig. 4.9). This suggests there may be a difference in investment in Rubisco protein content in species with different phloem loading strategies. There were significant species x [CO$_2$] and species x date x [CO$_2$] interactions, primarily due to changes in the passive loading species. Peony significantly increased its transition point at elevated [CO$_2$] compared to ambient on both dates in 2014 and strawberry significantly increased its transition point in May 2013. The transition point in apoplastic species was more stable.

At ambient [CO$_2$], photosynthesis for all of the apoplastic and passive loading species was Rubisco-limited (Fig. 4.10). At the elevated [CO$_2$] used in this experiment, photosynthesis was electron transport-limited in the apoplastic loading species and Rubisco-limited in passive loading species, due to their higher transition point (Fig. 4.10). This difference in limitation of photosynthesis could theoretically lead to differences in investment in Rubisco, since Rubisco is not limiting in the apoplastic species at elevated [CO$_2$]. However, there were no significant species x [CO$_2$] effects observed for $V_{c,max}$ (Table 4.2, Fig. 4.8).
Meta-Analysis of Photosynthetic Responses to Elevated [CO₂]

In this field study, there was no significant impact of phloem loading strategy on photosynthetic response to elevated [CO₂], but it only examined two species of each phloem loading type. To test whether phloem loading strategy impacted [CO₂] response across a wider range of species, a meta-analysis was performed of field elevated [CO₂] experiments on trees previously characterized as apoplastic or passive loading species. Because the phloem loading strategy for most species has not been explicitly characterized, the meta-analysis only included three apoplastic and four passive loading tree species and therefore was quite limited. \( V_{c,max} \) and \( J_{max} \) both significantly decreased in passive loading trees but not apoplastic loading species (Fig. 4.11) and there was a significant difference between the two phloem loading types. The between group heterogeneity (\( Q_b \)) for \( V_{c,max} \) was 10.31 with a p value of 0.001 and the number of studies (\( k \)) was 25. For \( J_{max} \), the \( Q_b \) was 6.14 with a p value of 0.01. The changes in \( V_{c,max} \) and \( J_{max} \) were small in this analysis (for \( V_{c,max} \), a 13% decrease in passive loading species and 6% increase in apoplastic loading species, for \( J_{max} \), a 11% decrease in passive loading species and a 2% increase in apoplastic loading species). Still, this result is counter to our original hypothesis, and also different from the results from the 4 herbaceous species measured in this study.

The decrease in photosynthetic capacity in passive loading trees did not translate to a significant difference in light-saturated photosynthesis (\( A_{sat} \)) between phloem loading types (Fig. 4.17, \( Q_b = 1.76, p = 0.18, k = 32 \)). Both apoplastic and passive loading trees had significant, large increases in photosynthesis at elevated [CO₂] (33.0% and 45.4% for passive and apoplastic loading trees, respectively).

Carbohydrate Responses to Elevated [CO₂]

Elevated [CO₂] did not consistently change total soluble sugar content in leaves of peony, berry beet and pea (Table 4.3, Fig. 4.12). Soluble sugar (glucose, fructose, and sucrose) content only increased with elevated [CO₂] in one of the two years. The increase in soluble sugars was generally small; only measurements at dawn in 2014 were significant when tested via pairwise comparisons. All species showed a diurnal progression in soluble sugar content with the greatest values measured at midday and dusk (Fig. 4.12). Strawberry and peony both had high soluble sugar content across all times of day, consistent with their passive method of loading phloem. Pea also had high soluble sugar content, about the same as the passive loading species, during the
day, dropping substantially during the night. Beet had a much lower soluble sugar content than the rest of the species at all times of day (Fig. 4.12).

Starch content was significantly increased at elevated [CO$_2$] across both years (Table 4.3, Fig. 4.13). Although some species, such as beet, had an accumulation of starch throughout the day which was used during the night, others, such as peony, did not appear to have any diurnal change in starch content, and so there was a significant species x time effect in both years. Since all species had an increase in photosynthesis at elevated [CO$_2$] and little decrease in photosynthetic capacity, it does not appear that the increase in starch negatively impacted photosynthesis at elevated [CO$_2$].

Carbon to nitrogen ratio significantly increased at elevated [CO$_2$] across both years (Table 4.4, Fig. 4.14 e, f). When averaged across both years, there was a 14% decrease in the percent nitrogen and a 7% decrease in nitrogen on a g/m$^2$ basis (Table 4.4, Fig. 4.14 a-d). There was no significant species x [CO$_2$] effect (Table 4.4). This only slight decrease in leaf nitrogen content is consistent with a fertilized field and the increased carbon: nitrogen ratio is consistent with the significant increase in carbon and starch at elevated [CO$_2$].

Changes in Leaf Anatomy at Elevated [CO$_2$]
There was a marginally significant increase in leaf thickness ($p = 0.0704$) in plants grown at elevated [CO$_2$], due primarily to an increase in spongy mesophyll thickness ($p = 0.0777$) (Table 4.5, Figs. 4.15, 4.16). The palisade mesophyll and epidermis thickness showed no significant difference at elevated [CO$_2$]. Despite the increase in spongy mesophyll thickness, there was no change in percent air space at elevated [CO$_2$] (Table 4.5). The increase in leaf thickness was consistent with the increase in specific leaf weight at elevated [CO$_2$] (Table 4.4, Fig. 4.14 g, h).

Pea leaf cross-sections were analyzed separately to determine if the length of the internal cell wall of transfer cells increased at elevated [CO$_2$]. This was expressed as a percent increase in the perimeter and area of the cell wall due to ingrowths. There was no significant increase in percent perimeter or area of cell wall ingrowths at elevated [CO$_2$] ($p = 0.1406, 0.3645$, respectively; Fig. 4.17).
Discussion

Elevated [CO₂] is predicted to increase plant photosynthesis, but the relative stimulation in photosynthesis can vary substantially between species, genotypes, and environmental conditions (Ainsworth & Long 2005; Hasegawa et al. 2013; Bishop et al. 2014; 2015). One of the major hypotheses for acclimation of photosynthesis at elevated [CO₂] in some species is sugar-mediated downregulation of photosynthesis (Moore et al. 1999; Long et al. 2004; Ainsworth & Rogers 2007). Previous studies have indicated that at elevated [CO₂], sugars can build up in the leaf and downregulate Rubisco, causing a decrease in photosynthetic capacity (Nie et al. 1995; Moore et al. 1998). In this study, however, there was a significant increase in diurnal photosynthetic C assimilation (Fig. 4.4, 4.5) and only a small decrease in V_c,max (Fig. 4.7). There was also little increase in soluble sugar content in leaves except at dawn in 2014 in pea (Fig. 4.12), indicating that there was little sugar-mediated feedback on photosynthesis. Davey et al. (2006) similarly observed strong stimulations in photosynthesis and no increase in soluble sugar in poplar grown at elevated [CO₂], attributing the response to poplar’s fast growth and strong sink strength. Other studies have observed increases in both soluble sugars and starch at elevated [CO₂] with little to no decrease in photosynthetic capacity (Ainsworth et al. 2004; Rogers et al. 2004; Ainsworth et al. 2007). Changes in starch content are less correlated with changes in photosynthetic capacity than soluble sugar content (Moore et al. 1998; Davey et al. 2006; DaMatta et al. 2016) and in this study, starch content increased dramatically in both years (Fig. 4.13). This study was performed in a field setting with no rooting limitation and there was little decrease in leaf nitrogen content (Fig. 4.14), so it appears that there was sufficient sink capacity in all species to maintain high stimulations in photosynthesis. Further studies growing these species in different sized pots or otherwise manipulating source: sink ratio could determine whether these results are consistent in scenarios with lower sink strength.

In addition to this field study analyzing the responses of two apoplastic and two passive loading species to elevated [CO₂], a meta-analysis was performed of field elevated [CO₂] experiments on trees previously characterized as apoplastic or passive loading species to give a wider range of species and functional types. Since so few species have been characterized by phloem loading strategy, the meta-analysis only included three apoplastic and four passive loading tree species, which severely limited the scope of the analysis. Therefore, although there was a significant difference between the two phloem loading types in both V_c,max and J_max (Fig. 4.16, 4.17).
4.11), more experiments would need to be done and more species tested to perform a broader meta-analysis of the impacts of phloem loading strategy on photosynthetic response to elevated [CO₂] in trees.

One significant difference between phloem loading types was the transition point between Rubisco- and electron transport-limited photosynthesis. The passive loading species had significantly higher transition points across all measurement dates (Fig. 4.9) and were therefore Rubisco-limited at both ambient and elevated [CO₂] (Fig. 4.10). Apoplastic loading species, in contrast, were Rubisco-limited at ambient [CO₂], but electron transport-limited at elevated [CO₂] (Fig. 4.10). Few comparisons of transition point between species have been published. In his meta-analysis of published A/cᵢ data, Wullschleger (1993) assumed that the transition point was constant between species and at a cᵢ of approximately 20-25 Pa. This is lower than the transition point for both the apoplastic loading (33-34 Pa) and passive loading (50-53 Pa) species analyzed in this experiment. Manter et al. (2004) took measurements of A vs cᵢ in 19 tree species and found a range of transition points between 25 and 152 Pa for conifers and 20 and 78 Pa for broadleaved species. A systematic characterization of transition point across a wider range of species and functional types has not yet been performed, but would be useful to determine if these differences in transition point across phloem loading strategy are consistent across more species.

Since passively loading species require a concentration gradient to load their phloem, they are hypothesized to have higher mesophyll sucrose concentrations than apoplastic loading species (Rennie & Turgeon 2009; Turgeon 2010). In fact, one of the hypotheses for the evolution of apoplastic phloem loading is to decrease leaf carbohydrates and increase growth potential (Turgeon 2010). In this analysis, pea, an apoplastic loading species, had as high concentrations of soluble sugars as passive loading species (Fig. 4.12) during the day. Huber (1989) and Kingston-Smith et al. (1998) observed that pea tended to accumulate more sucrose in the leaves than other species and had a low acid invertase activity. Pea therefore has been characterized as a ‘sucrose storer’, while beet has been characterized as a ‘starch storer’ (Huber 1989; Goldschmidt & Huber 1992). Kingston-Smith et al. (1998) found that across thirteen species, two symplastic loading species, poplar and grape, had the highest concentrations of foliar sucrose, but the other two symplastic loading species, Fuchsia and Hydrangea, had similar levels to apoplastic loading species. This study used anatomy alone to differentiate phloem
loading types, so symplastic loading could include polymer trapping or passive loading species. Poplar and grape exhibited high dawn sucrose concentrations, unlike most apoplastic loading species, which had very low levels of sucrose at dawn even if they tended to store sucrose rather than starch during the day (Kingston-Smith et al. 1998), consistent with the findings of this study (Fig. 4.12). The diurnal progression supports the hypothesis that peony and strawberry require high sucrose concentrations at all times to passively load their phloem (Rennie & Turgeon 2009), while pea and other sucrose storing apoplastic loading species use sucrose as carbon storage during the day and sucrose concentration is not tied to phloem loading ability.

Elevated [CO₂] has been shown to alter leaf anatomy, including leaf thickness (Pritchard et al. 1999; Engloner et al. 2003; Xu et al. 2012). In this study, leaf thickness marginally increased at elevated [CO₂], primarily due to an increase in spongy mesophyll thickness (Figs. 4.15, 4.16), as Xu et al. (2012) observed in *Eucalyptus*. Increasing source capacity by high light conditions was previously shown to change the anatomy of pea transfer cells by increasing cell wall invaginations (Amiard et al. 2005; Adams et al. 2007). This change in anatomy was not observed at elevated [CO₂] (Fig. 4.17), perhaps because the change in source capacity between the ambient and elevated [CO₂] used in this study is not as drastic as the change in source capacity between their low light (150 PPFD) and high light (1000 PPFD) treatments. More controlled conditions or a much higher elevated [CO₂] may elucidate whether the increase in cell wall invaginations associated with higher source capacity can occur in elevated [CO₂].

In conclusion, this study found no significant effect of phloem loading strategy on photosynthetic response to elevated [CO₂]. All species had strong stimulations in diurnal photosynthesis and little decrease in photosynthetic capacity. This suggests that there was sufficient sink strength in all species at elevated [CO₂], regardless of sucrose transport mechanism, and that sugar-mediated downregulation of photosynthesis at elevated [CO₂] may not occur in all circumstances.
### TABLE 4.1

Table 4.1. Analysis of variance of the response of photosynthesis and stomatal conductance of four species exposed to ambient and elevated [CO₂]. Results from the analysis of variance (F test) and statistical significance (P) for each source of variation are shown. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th>May 2013</th>
<th>June 2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthesis (A)</td>
<td>Stomatal Conductance (gₛ)</td>
</tr>
<tr>
<td>Species (S)</td>
<td>408, &lt;0.0001</td>
</tr>
<tr>
<td>CO₂</td>
<td>90, &lt;0.0001</td>
</tr>
<tr>
<td>S x CO₂</td>
<td>7.19, 0.002</td>
</tr>
<tr>
<td>Time</td>
<td>58, &lt;0.0001</td>
</tr>
<tr>
<td>Time x S</td>
<td>18.2, &lt;0.0001</td>
</tr>
<tr>
<td>Time x</td>
<td>3.58, 0.01</td>
</tr>
<tr>
<td>CO₂</td>
<td>Time x S x</td>
</tr>
<tr>
<td>LiCor</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>May 2014</th>
<th>June 2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species (S)</td>
<td>186, &lt;0.0001</td>
</tr>
<tr>
<td>CO₂</td>
<td>179, &lt;0.0001</td>
</tr>
<tr>
<td>S x CO₂</td>
<td>6.10, 0.0009</td>
</tr>
<tr>
<td>Time</td>
<td>131, &lt;0.0001</td>
</tr>
<tr>
<td>Time x S</td>
<td>7.1, &lt;0.0001</td>
</tr>
<tr>
<td>Time x</td>
<td>1.66, 0.17</td>
</tr>
<tr>
<td>CO₂</td>
<td>Time x S x</td>
</tr>
<tr>
<td>LiCor</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4.2. Analysis of variance of the response of photosynthetic characteristics of four species exposed to ambient and elevated [CO₂]. This includes the integral of diurnal photosynthesis (A’), maximum carboxylation rate (Vc,max), maximum rate of electron transport (Jmax), and the ci at which photosynthesis transitioned from Rubisco-limited to electron transport-limited (transition point). Results from the analysis of variance (F test) and statistical significance (P) for each source of variation are shown. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>A’</th>
<th>Vc,max</th>
<th>Jmax</th>
<th>Transition Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species (S)</td>
<td>587, &lt;0.0001</td>
<td>196, &lt;0.0001</td>
<td>111, &lt;0.0001</td>
<td>89, &lt;0.0001</td>
</tr>
<tr>
<td>CO₂</td>
<td>478, &lt;0.0001</td>
<td>5.69, 0.02</td>
<td>2.94, 0.09</td>
<td>8.93, 0.0041</td>
</tr>
<tr>
<td>S x CO₂</td>
<td>9.84, &lt;0.0001</td>
<td>0.43, 0.73</td>
<td>0.06, 0.98</td>
<td>2.94, 0.0403</td>
</tr>
<tr>
<td>Date</td>
<td>16.2, &lt;0.0001</td>
<td>9.42, &lt;0.0001</td>
<td>5.91, 0.0013</td>
<td>8.82, &lt;0.0001</td>
</tr>
<tr>
<td>Date x S</td>
<td>7.69, &lt;0.0001</td>
<td>2.19, 0.04</td>
<td>1.34, 0.24</td>
<td>2.15, 0.04</td>
</tr>
<tr>
<td>Date x CO₂</td>
<td>1.66, 0.18</td>
<td>0.93, 0.43</td>
<td>1.30, 0.28</td>
<td>1.01, 0.40</td>
</tr>
<tr>
<td>Date x S x CO₂</td>
<td>0.79, 0.61</td>
<td>0.65, 0.74</td>
<td>1.89, 0.08</td>
<td>2.84, 0.01</td>
</tr>
</tbody>
</table>
Table 4.3. Analysis of variance of the response of soluble sugars and starch of four species exposed to ambient and elevated [CO₂]. Results from the analysis of variance (F test) and statistical significance (P) for each source of variation are shown. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th></th>
<th>Total Soluble Sugars</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>June 2013</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species (S)</td>
<td>171, &lt;0.0001</td>
<td>96, &lt;0.0001</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.07, 0.79</td>
<td>50, &lt;0.0001</td>
</tr>
<tr>
<td>S x CO₂</td>
<td>0.59, 0.63</td>
<td>2.26, 0.12</td>
</tr>
<tr>
<td>Time</td>
<td>58, &lt;0.0001</td>
<td>2.21, 0.10</td>
</tr>
<tr>
<td>Time x S</td>
<td>6.96, &lt;0.0001</td>
<td>6.02, &lt;0.0001</td>
</tr>
<tr>
<td>Time x CO₂</td>
<td>0.41, 0.75</td>
<td>2.24, 0.10</td>
</tr>
<tr>
<td>Time x S x CO₂</td>
<td>0.70, 0.70</td>
<td>1.38, 0.22</td>
</tr>
<tr>
<td><strong>June 2014</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species (S)</td>
<td>236, &lt;0.0001</td>
<td>14.5, 0.0005</td>
</tr>
<tr>
<td>CO₂</td>
<td>20, 0.0007</td>
<td>11.6, 0.005</td>
</tr>
<tr>
<td>S x CO₂</td>
<td>3.88, 0.05</td>
<td>4.36, 0.04</td>
</tr>
<tr>
<td>Time</td>
<td>63, &lt;0.0001</td>
<td>24, &lt;0.0001</td>
</tr>
<tr>
<td>Time x S</td>
<td>9.33, 0.0001</td>
<td>2.63, 0.04</td>
</tr>
<tr>
<td>Time x CO₂</td>
<td>3.91, 0.03</td>
<td>0.15, 0.93</td>
</tr>
<tr>
<td>Time x S x CO₂</td>
<td>3.33, 0.03</td>
<td>2.59, 0.05</td>
</tr>
</tbody>
</table>
Table 4.4. Analysis of variance of the response of leaf nitrogen, carbon to nitrogen ratio, and specific leaf weight of four species exposed to ambient and elevated \([\text{CO}_2]\). Samples were taken at midday on June 19, 2013 and June 17, 2014. Results from the analysis of variance (F test) and statistical significance (P) for each source of variation are shown. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Leaf Nitrogen (%)</th>
<th>Leaf Nitrogen (g/m²)</th>
<th>Carbon: Nitrogen</th>
<th>Specific Leaf Weight (g/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species (S)</td>
<td>115, &lt;0.0001</td>
<td>26, &lt;0.0001</td>
<td>201, &lt;0.0001</td>
<td>89.74, &lt;0.0001</td>
</tr>
<tr>
<td>(\text{CO}_2)</td>
<td>20, 0.0001</td>
<td>6.20, 0.02</td>
<td>23, &lt;0.0001</td>
<td>6.27, 0.02</td>
</tr>
<tr>
<td>S x (\text{CO}_2)</td>
<td>0.20, 0.89</td>
<td>0.48, 0.70</td>
<td>0.81, 0.50</td>
<td>0.44, 0.73</td>
</tr>
<tr>
<td>Date</td>
<td>6.60, 0.02</td>
<td>1.46, 0.24</td>
<td>8.40, 0.007</td>
<td>2.18, 0.15</td>
</tr>
<tr>
<td>Date x S</td>
<td>3.88, 0.03</td>
<td>6.32, 0.005</td>
<td>3.66, 0.04</td>
<td>0.30, 0.75</td>
</tr>
<tr>
<td>Date x (\text{CO}_2)</td>
<td>2.91, 0.10</td>
<td>0.53, 0.47</td>
<td>1.67, 0.21</td>
<td>4.22, 0.05</td>
</tr>
<tr>
<td>Date x S x (\text{CO}_2)</td>
<td>0.15, 0.86</td>
<td>0.46, 0.63</td>
<td>0.03, 0.97</td>
<td>0.40, 0.68</td>
</tr>
</tbody>
</table>
Table 4.5. Analysis of variance of the response of leaf anatomy of four species exposed to ambient and elevated [CO₂]. Samples were taken on June 25, 2014. Results from the analysis of variance (F test) and statistical significance (P) for each source of variation are shown. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>[CO₂]; F, p</th>
<th>Species</th>
<th>[CO₂] x Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Thickness</td>
<td>3.76, 0.0704</td>
<td>91.05, &lt;0.0001</td>
<td>0.70, 0.5660</td>
</tr>
<tr>
<td>Palisade Thickness</td>
<td>0.02, 0.8774</td>
<td>37.30, &lt;0.0001</td>
<td>0.20, 0.8177</td>
</tr>
<tr>
<td>Spongy Thickness</td>
<td>3.72, 0.0777</td>
<td>316.14, &lt;0.0001</td>
<td>0.63, 0.5496</td>
</tr>
<tr>
<td>Upper Epidermis</td>
<td>0.27, 0.6102</td>
<td>114.25, &lt;0.0001</td>
<td>1.12, 0.3721</td>
</tr>
<tr>
<td>Lower Epidermis</td>
<td>1.65, 0.2173</td>
<td>40.13, &lt;0.0001</td>
<td>1.29, 0.3115</td>
</tr>
<tr>
<td>Percent Air Space</td>
<td>0.61, 0.4445</td>
<td>76.16, &lt;0.0001</td>
<td>0.68, 0.5793</td>
</tr>
</tbody>
</table>
Figure 4.1. Layout of the miniFACE site. Species in each plot are from 2013. In 2014, species were randomized again inside the plots, but the [CO₂] of each plot was the same as in 2013. Diagram is not to scale.
Figure 4.2. Ten minute average fumigation data for a sample week from (a) 2013 and (b) 2014. Ambient [CO₂] data was taken from outside plot 6. Fumigation for the “master plot” (plot 3, in green) was more consistent and centered around 600 ppm than the other two plots (plots 1 and 5, in red and blue, respectively).
Figure 4.3. Planting diagram for a sample FACE plot. Diagram is to scale.
Figure 4.4. Photosynthesis ($A$) measured throughout the day for four dates across two growing seasons on ambient (white) and elevated (black) [CO$_2$] grown (a-d) peony, (e-h) strawberry, (i-k) beet, and (l-o) pea. Asterisk(s) indicate significant effects for [CO$_2$] for each species and time period tested with linear contrasts ($(*) p < 0.10$, $* p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Each symbol represents the mean ($\pm$ standard error) of three plots, except peony at elevated [CO$_2$] in 2014, which represents the mean of two plots.
Figure 4.5. The integral of daily photosynthesis (A') for ambient [CO₂] (white bars) or elevated [CO₂] (black bars) grown (a) peony, (b) berry, (c) beet, and (d) pea for four time periods during the two growing seasons. Bars with asterisk(s) indicate significant effects for [CO₂] for each species and time period tested with linear contrasts ( (*) p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001). Percent stimulation is shown above each bar as the ratio of the elevated [CO₂] value to the ambient [CO₂] value. Each symbol represents the mean (± standard error) of three plots except the elevated [CO₂] treatment in peony in 2014, which is the mean of two plots.
Figure 4.6. Stomatal conductance ($g_s$) measured throughout the day for four dates across two growing seasons on ambient (white) and elevated (black) [CO$_2$] grown (a-d) peony, (e-h) strawberry, (i-k) beet, and (l-o) pea. Asterisk(s) indicate significant effects for [CO$_2$] for each species and time period tested with linear contrasts ( (*) p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001). Each symbol represents the mean (± standard error) of three plots, except peony at elevated [CO$_2$] in 2014, which represents the mean of two plots.
Figure 4.7. The maximum carboxylation rate ($V_{c,\text{max}}$) of ambient [CO$_2$] (white bars) or elevated [CO$_2$] (black bars) grown (a) peony, (b) strawberry, (c) beet, and (d) pea from four dates across the two growing seasons. Bars with asterisk(s) indicate significant effects for [CO$_2$] for each species and time periods tested with linear contrasts ( * p < 0.10, * p < 0.05). Percent stimulation is shown above each bar as the ratio of the elevated [CO$_2$] value to the ambient [CO$_2$] value. Each symbol represents the mean (± standard error) of three plots.
Figure 4.8. The maximum electron transport rate ($J_{\text{max}}$) of ambient [CO$_2$] (white bars) or elevated [CO$_2$] (black bars) grown (a) peony, (b) strawberry, (c) beet, and (d) pea from four dates across the two growing seasons. Bars with asterisk(s) indicate significant effects for [CO$_2$] for each species and time periods tested with linear contrasts ( (*) $p < 0.10$, * $p < 0.05$). Percent stimulation is shown above each bar as the ratio of the elevated [CO$_2$] value to the ambient [CO$_2$] value. Each symbol represents the mean (± standard error) of three plots.
Figure 4.9. The $c_i$ value at the transition point between Rubisco limited and electron transport limited photosynthesis in the A/$c_i$ curve in ambient [CO$_2$] (white bars) or elevated [CO$_2$] (black bars) grown (a) peony, (b) strawberry, (c) beet, and (d) pea from four dates across the two growing seasons. Bars with asterisk(s) indicate significant effects for [CO$_2$] for each species and time periods tested with linear contrasts ( (*) p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001). Percent stimulation is shown above each bar as the ratio of the elevated [CO$_2$] value to the ambient [CO$_2$] value. Each symbol represents the mean (± standard error) of three plots except the ambient [CO$_2$] grown peony in June 2014, which represents the mean of two plots.
Figure 4.10. Photosynthesis ($A$) as a function of internal $[\text{CO}_2]$ ($C_i$) in (a) peony, (b) strawberry, (c) beet, and (d) pea. Curves were generated from $V_{c,max}$, $J_{max}$, and $R_d$ values averaged over all measurements using the equations of Farquhar et al. (1980) from ambient (blue) and elevated (red) curves. Dashed lines indicate the supply function for each growth $[\text{CO}_2]$ (short dash = ambient $[\text{CO}_2]$, dash-dot = elevated $[\text{CO}_2]$). Arrows indicate the transition point between Rubisco limited and electron transport limited photosynthesis.
Figure 4.11. The effect of phloem loading strategy on (a) $V_{c,max}$, (b) $J_{max}$, and (c) light saturated photosynthesis ($A_{sat}$) in tree species. Symbols represent percent change at elevated [CO$_2$] and 95% confidence intervals are shown. Degrees of freedom for each confidence interval are given on the right side.
Figure 4.12. Total soluble sugar (TSS; glucose + fructose + sucrose) content measured throughout the day on (a, b, d, f) June 2013 and (c, e, g) June 2014 in ambient (white) and elevated (black) \([\text{CO}_2]\) grown (a) peony, (b-c) strawberry, (d-e) beet, and (f-g) pea. Asterisk(s) indicate significant effects for \([\text{CO}_2]\) for each species and time periods tested with linear contrasts (\(*\) \(p < 0.10\), * \(p < 0.05\), ** \(p < 0.01\)). Each symbol represents the mean (± standard error) of three plots.
Figure 4.13. Starch content measured throughout the day on (a, b, d, f) June 2013 and (c, e, g) June 2014 in ambient (white) and elevated (black) [CO₂] grown (a) peony, (b-c) strawberry, (d-e) beet, and (f-g) pea. Asterisk(s) indicate significant effects for [CO₂] for each species and time periods tested with linear contrasts ( (*) p < 0.10, * p < 0.05, ** p < 0.01, *** p < 0.001). Each symbol represents the mean (± standard error) of three plots.
Figure 4.14. Leaf nitrogen (a-d), carbon to nitrogen ratio (e-f), and specific leaf weight (g-h) of four species exposed to ambient and elevated [CO₂]. Samples were taken at midday on June 19, 2013 and June 17, 2014. Bars with asterisk(s) indicate significant effects for [CO₂] for each species and time periods tested with linear contrasts ( (*) p < 0.10, * p < 0.05, ** p < 0.01). Each symbol represents the mean (± standard error) of three plots.
Figure 4.15. Leaf anatomical characteristics of four species grown at ambient [CO$_2$] (white bars) or elevated [CO$_2$] (black bars): (a) leaf thickness, (b) percent air space, (c) palisade mesophyll thickness, (d) spongy mesophyll thickness, (e) upper epidermis thickness, and (f) lower epidermis thickness. Samples were taken on June 25, 2014. Bars with asterisk(s) indicate significant effects for [CO$_2$] for each species and time periods tested with linear contrasts ( (* p < 0.10, * p < 0.05). Each symbol represents the mean (± standard error) of three plots.
Figure 4.16. Light microscope images of leaf tissue. Peony (a, b), strawberry (c, d), beet (e, f), and pea (g, h) were grown at ambient (a, c, e, g) or elevated [CO₂] (b, d, f, h).
Figure 4.17. Transmission electron microscope images of pea minor veins. Plants were grown at (a) ambient or (b) elevated [CO₂]. There was no significant difference in the percent increase in perimeter due to cell wall invaginations (p = 0.1406) or percent of area devoted to cell wall invaginations (p = 0.3645) between ambient and elevated [CO₂]. TC, transfer cell; SE, sieve element.
CHAPTER V: IMPACTS OF CHANGING SUCROSE TRANSPORTER EXPRESSION ON ELEVATED [CO₂] RESPONSE IN ARABIDOPSIS THALIANA

Abstract
When the capacity for leaves to produce carbohydrates exceeds the capacity of sinks to utilize carbohydrates, sugars can accumulate in leaves and negatively feedback on photosynthesis. One strategy for circumventing this negative feedback may be to increase sucrose transporter expression and activity in the companion cells of phloem. In this study, Arabidopsis thaliana plants over-expressing sucrose transporters were grown at elevated [CO₂] in order to test the hypothesis that enhanced transport expression would improve photosynthetic and growth response in an environment of high source capacity. Two different transgenic constructs were tested. In the first (HvSUT1), a barley sucrose transporter with increased transport activity and specificity replaced the native AtSUC2 gene, and in the second (AtSUC1) the viral companion cell-specific CoYMV promoter was used to overexpress the native AtSUC1 protein. In contrast to the hypothesis, there was no evidence that overexpression of sucrose transporters increased photosynthetic capacity in either ambient or elevated [CO₂], since both HvSUT1 and AtSUC1 had similar photosynthetic capacity to wild-type. However, AtSUC1 plants showed dramatically reduced growth rates, especially at ambient [CO₂] compared to wild-type and HvSUT1. In previous experiments with AtSUC1, apparent phosphate limitation was proposed to explain the reduced growth. In these experiments, however, transcriptional evidence for phosphate limitation was not observed. Further studies may be needed to determine why there was a decrease in biomass with no perceived phosphate limitation or why the AtSUC1 plants had such a large stimulation in biomass at elevated [CO₂].

Introduction
One of the primary purposes of phloem is transporting sucrose generated by source (leaves) tissue to sink (roots, flowers, fruits) tissue (Ainsworth & Bush 2011; Yadav et al. 2015). In apoplastic loading species, sucrose generated by mesophyll cells is pumped into the apoplast using SWEET transporters (Chen 2014). This sucrose is then pumped into the companion cells of the phloem using proton motive force via proton-coupled sucrose symporters (SUTs; Lalonde et al. 2004; Yadav et al. 2015). SWEETs are localized to the phloem parenchyma cells (Chen et
al. 2012), while SUTs are localized to the companion cells of the sieve elements and load sucrose from the apoplast into the companion cells of the conducting sieve elements against a concentration gradient (Gottwald et al. 2000). Sucrose then moves via mass flow from phloem in source tissue to sink tissues in the plant.

Previous studies have shown that source and sink capacities are directly related to photosynthetic capacity (Plaut et al. 1987; Krapp & Stitt 1995; Paul & Foyer 2001). Higher CO₂ concentration ([CO₂]) can increase source supply, and [CO₂] has increased from 280 ppm to about 400 ppm since the Industrial Revolution and will continue to increase given current emissions trends (Ciais et al. 2013). Elevated [CO₂] directly stimulates light-saturated, net photosynthetic CO₂ uptake (A) in C₃ plants, but can also lead to a buildup of carbohydrates in the leaf, especially when sink capacity is limited (Moore et al. 1998; Rogers et al. 2004; Ainsworth & Long 2005; Aranjuelo et al. 2013). Excess carbohydrates in leaves are hypothesized to negatively feedback on Rubisco expression and content through either intracellular or extracellular invertase activity (Moore et al. 1999). Elevated [CO₂] also increases the starch to sucrose ratio, and can increase nighttime glucose concentrations as a result of starch and maltose metabolism at night, which could negatively feedback on photosynthetic capacity (Sharkey et al. 2004). Because of the negative feedback of carbohydrates on photosynthetic capacity, increasing sucrose transport has been hypothesized to increase photosynthesis and biomass at elevated [CO₂] (Ainsworth & Bush 2011; Stitt 2013).

Increasing sucrose transporter expression or altering the major sucrose transporter responsible for phloem loading has been performed in potato (Solanum tuberosum: Leggewie et al. 2003), rice (Oryza sativa; Wang et al. 2015), and Arabidopsis thaliana (Reinders et al. 2012; Wippel & Sauer 2012; Dagupta et al. 2014). Wippel & Sauer (2012) replaced SUC2, the sucrose transporter primarily responsible for phloem loading in Arabidopsis thaliana (Srivastava et al. 2009a), with the Arabidopsis SUC1. AtSUC2 and AtSUC1 are both type I sucrose transporters (Reinders et al. 2012) and have similar activities, but slightly different pH ranges (Sauer et al. 1994). AtSUC2 is primarily expressed in leaves (Sauer et al. 1994), while AtSUC1 is primarily expressed in pollen, roots, and trichomes (Sivitz et al. 2007). When AtSUC1 was driven by the AtSUC2 promoter in a suc2 knockout line, the plants were successfully complemented, indicating that AtSUC1 can act as an effective transporter for phloem loading (Wippel & Sauer 2012). In two of the lines, sucrose and glucose concentrations in the leaf were significantly
lower, potentially indicating that the AtSUC1 transporter could be more efficient. Dasgupta et al. (2014) also expressed AtSUC1 using a viral companion cell specific promoter CoYMV (Srivastava et al. 2009b) in a suc2 knockout line. The plants were almost as large as wildtype so the CoYMV promoter driving SUC1 complimented the phenotype. CoYMV expression was localized to companion cells in the leaves, stem, and roots when expressed in tobacco (Matsuda et al. 2002). When Reinders et al. (2012) replaced SUC2 with the barley (Hordeum vulgare) sucrose transporter HvSUT1, the new transporter also complemented the phenotype and produced a similar sized plant. No further study of this line, however, has been published.

HvSUT1 is a type II sucrose transporter and has higher specificity for sucrose compared to other sugars as well as higher transport activity for sucrose than AtSUC2 when expressed in Xenopus oocytes (Sivitz et al. 2005).

Although changing the activity of sucrose transporters may alter phloem loading capacity slightly, overexpressing them, particularly using a companion cell-specific promoter, was hypothesized to increase productivity even more (Ainsworth & Bush 2011; Stitt 2013). The beet type I sucrose transporter BvSUT1 was determined to be primarily transcriptionally regulated via a phosphorylation pathway (Vaughn et al. 2002; Ransom-Hodgkins et al. 2003), so it is hypothesized that increasing sucrose transporter expression would increase overall sucrose transporter activity. Leggewie et al. (2003) expressed the spinach SoSUT1 sucrose transporter in potato using the constitutive 35S promoter. They found no effect of increasing sucrose transporter expression on photosynthesis or tuber yield, but leaves showed lower soluble sugar (glucose, fructose, and sucrose) content and altered starch metabolism. Tubers had higher soluble sugar content. Since the 35S promoter is expressed in all cells, futile cycling may have limited the benefit from increased sucrose transport (Leggewie et al. 2003). Wang et al. (2015) observed an increase in biomass and yield by expressing AtSUC2 in rice grown in the field with the companion cell specific promoter PP2. In addition, they found an increase in sucrose export in the phloem, but no change in leaf soluble sugars or photosynthesis.

Dasgupta et al. (2014) used the companion cell specific viral promoter CoYMV to drive AtSUC1, AtSUC2, or the maize sucrose transporter ZmSUT1 in wild-type plants to increase sucrose transport capacity. Unlike the AtSUC2 promoter, which is repressed when the plant is grown with greater external sucrose (Osuna et al. 2007; Dasgupta et al. 2014), the CoYMV promoter is activated when the plant is grown with greater external sucrose (Dasgupta et al.
Overexpressing sucrose transporters was hypothesized to increase photosynthesis and biomass, although in fact, biomass was decreased in all transgenic lines despite the increase in sucrose export from the leaves. The decrease in biomass was accompanied by an increase in the expression of genes related to phosphate limitation (Dasgupta et al. 2014). Increases in sucrose transport have previously been related to increases in perceived phosphate limitation (Hammond & White 2008; Lei et al. 2011) and greater stunting of growth when phosphorus is limiting.

When plants with altered sucrose transporter expression were grown at low light and at ambient [CO₂] in growth chambers or greenhouses, plant growth was either stunted or unchanged (Leggewie et al. 2003; Dasgupta et al. 2014). In contrast, when other lines with manipulated sucrose transporter expression were grown in the field, there was stimulation or no change in plant growth (Leggewie et al. 2003; Wang et al. 2015). Initial hypotheses had suggested that the benefit from an increase in sucrose transporter expression or activity would be greater in times of high source capacity, such as at elevated [CO₂] or high light, since sugars would be more likely to build up in the leaf in these scenarios (Ainsworth & Bush 2011; Stitt 2013). The perceived phosphate limitation observed by Dasgupta et al. (2014) in sucrose transporter overexpressing plants, however, could potentially limit the benefit of increased sucrose transport regardless of light or [CO₂], especially if plants at elevated [CO₂] require more phosphate (Jin et al. 2015).

This study used high light growth chamber conditions and elevated [CO₂] to test the prediction that increasing or changing sucrose transporter expression would enhance the photosynthetic and biomass response to elevated [CO₂] due to less sugar mediated downregulation of photosynthesis. The HvSUT1 genotype, which replaced the native AtSUC2 sucrose transporter with the more specific and active HvSUT1 sucrose transporter, driven by the native AtSUC2 promoter, was predicted to have a slightly greater benefit from elevated [CO₂] than wild-type due to the greater activity of the HvSUT1 transporter. The AtSUC1 genotype was predicted to have an even greater stimulation in biomass and photosynthesis at elevated [CO₂], since in this line, the native AtSUC2 expression was kept but additional sucrose transporter expression was driven by CoYMV, a companion cell-specific promoter activated by sucrose. Therefore, this construct may be able to enhance sucrose transport even further when sucrose transport may be limiting at elevated [CO₂].
**Materials and Methods**

*Plant Material and Growth Conditions*

Three lines of *A. thaliana* were used for this experiment, all of which were ecotype Columbia. One transgenic line, designated HvSUT1, was constructed similarly to that previously described in Reinders et al. (2012), except that this line was produced in a Columbia background and with the *atsuc2-5* mutant rather than the *atsuc2-1* mutant. Briefly, *atsuc2-5* mutant plants were complimented with the HvSUT1 gene (CAJ20123.1), using the AtSUC2 (At1g22710) promoter and 3’ UTR and the constructs were assembled into the pB7m34GW binary vector (Karimi et al. 2005). The other transgenic line, designated AtSUC1, was constructed by Dasgupta et al. (2014). Briefly, the CoYMV promoter (Srivastava et al. 2009) was used to drive the AtSUC1 gene (NM_105846) in a wild-type background. This line was designated At1-4-4 in Dasgupta et al. (2014) and used the construct pGPTV-CoyYMVP::cSUC1::CmR-ccdB. All plants were genotyped and found to be homozygous for the respective transgenes.

For the experiment, *A. thaliana* seeds were sterilized and vernalized in ddH2O for three days prior to planting. After vernalization, they were planted directly into sterilized soil (LC1 Sunshine Mix, SunGro Horticulture, Agawam, MA, USA) and grown in two growth chambers (Conviron PGR14, Controlled Environments Ltd, Winnpeg, Manitoba, Canada) in 514 mL pots. Plants were grown in a 10h/14h day/night schedule at 650 µmol m⁻² s⁻¹ PPFD, 70% RH, and 21°C day / 18°C night conditions. CO₂ fumigation began the day after planting and the concentrations of the ambient and elevated CO₂ treatments were 450 and 800 ppm, respectively. Fumigation was monitored every two minutes and the values were within 10% of the set point for 95.8% of the time. Plants were rotated once every two days in the chamber and rotated between chambers every five days to prevent chamber effects. They were watered alternately with a 40% Long Ashton solution or distilled water every 3-4 days. Plants were thinned to one plant per pot at 12 days after planting (DAP).

*Photosynthesis and Carbohydrate Measurements*

At 36 DAP for wild-type and HvSUT1 plants and 41 DAP for AtSUC1 plants, A/cᵢ curves were measured on youngest fully expanded leaves of 5 plants per genotype and treatment using the same protocol as in Chapter 4, except that plants were measured in the laboratory rather than in the field. Four portable open gas-exchange systems (LI-6400, Li-Cor Inc, Lincoln, NE, USA)
were used. The AtSUC1 plants were measured at a later date due to differences in development between genotypes. Measurements were performed at 1500 µmol m⁻² s⁻¹ PPFD and the block temperature was set at 25°C. $V_{c,max}$, $J_{max}$, the transition point, and dark respiration ($R_d$) were determined using the equations of Farquhar et al. (1980). All values were corrected to 25°C using the temperature corrections of Bernacchi et al. (2001; 2003), although little variation in temperature was observed.

The day after $A/c_i$ curves were performed (37 DAP for wild-type and HvSUT1, 42 DAP for AtSUC1), leaf disks (13.4 mm diameter) for carbohydrate analysis were collected 6.5 hours into the light period from eight plants per genotype and treatment, and were immediately frozen in liquid nitrogen. These leaf disks were from youngest fully expanded leaves. These samples were analyzed for glucose, fructose, and sucrose content using the methods of Jones et al. (1977). Starch was extracted and digested and content was determined as glucose equivalents using the methods of Hendricks et al. (2003). One leaf of approximately the same age was taken for specific leaf area at the same time by excising it at the petiole and scanning it. The area was determined using ImageJ version 1.48 ([http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) and dry weight was determined after one week of oven drying at 70°C.

**RNA Expression Analysis**

Another young fully expanded leaf per plant (n = 8) was taken at the same time as the carbohydrate samples for quantitative real-time PCR to confirm SUT overexpression and determine the presence of perceived phosphate limitation. Two leaves from two different plants of the same genotype and treatment were pooled for each sample, giving a total n of 4. Leaf tissue was ground in liquid nitrogen and RNA was extracted using PureLink® Plant RNA Reagent (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Potential genomic DNA contamination was removed using DNA-free DNase treatment (Applied Biosystems/Ambion, Austin, TX, USA). RNA concentration and quality were determined respectively using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA). cDNA were synthetized from 1 µg of RNA using Superscript II Reverse Transcriptase

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6 Gene expression analysis was performed by Pauline Lemonnier.
(Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. The qRT-PCR experiment was performed by using a 7900 HT Fast real-time PCR system (Applied Biosystems, Waltham, MA, USA). For each sample, three technical replicates were loaded in 384-well plates. The PCR mix consisted of a final volume of 10 µl containing X1 Power SYBRGreen® PCR Master Mix (Applied Biosystems, Waltham, MA, USA), 0.4 µM forward and reverse primers, and 2 µl of 10-time diluted cDNA. All primer sequences were taken from previous publications (Czechowski et al., 2005; Dasgupta et al. 2014), except for the HvSUT1 primers, which were designed using Primer 3 v. 0.4.0 (Untergrasser et al. 2012; Table 5.1) The amplification program was composed of an activation stage of 2 min at 50°C, a denaturation stage of 10 min at 95°C, and 40 amplification cycles with a 15-sec stage at 95°C and a 1-min step at 60°C. This was followed by a dissociation stage where the temperature was raised to 95°C for 15 sec then decreased to 60°C for 15 sec and gradually increased back to 95°C. Primer efficiency (E) and threshold cycle (Ct) values were provided by the SDS software (Applied Biosystems, Waltham, MA, USA) and the expression levels of genes of interest were determined using the method described by Schmittgen & Livak (2008).

Harvest, Leaf Area, and Statistics

Ten plants per genotype and treatment were measured every three to four days for leaf number and maximum rosette diameter from 8 DAP until 37 DAP. Pictures of plants of all genotypes were taken approximately 15 cm above the plant canopy using an EasyShare c180 camera (Kodak, Rochester, NY, USA) at 32 DAP. The visible leaf area was determined using ImageJ by tracing the rosette and determining the area inside.

All plants per genotype and treatment were harvested to determine leaf number, aboveground and belowground biomass at 39-40 DAP for wild-type and HvSUT1 plants and 44 DAP for AtSUC1 plants. Leaves were cut off at the base and leaves that were larger than 0.8 cm in length were counted. Leaves, flowering stalks, and roots were separated before drying. Roots were washed with water to separate them from the soil. All harvest samples were dried at 70°C for at least one week and weighed. In total, 14-18 plants were harvested per genotype and treatment.

All measured parameters were analyzed with a two-way analysis of variance (PROC MIXED, SAS 9.3; SAS Institute, Cary, NC, USA), with [CO2] and species as fixed effects.
Significant differences (p < 0.05) between genotypes and CO₂ treatments were determined through the pdiff LSMeans statement in SAS with no adjustment. When necessary, data was transformed to fit the requirements of the ANOVA. A log transformation was used for carbohydrates, AtSUC1, AtSUC2, and PAP24 expression, root weight, and flower weight. A square root transformation was used for PAP14 expression. A point was discarded as an outlier if the residual was more than three times the interquartile range of the residuals.

**Results and Discussion**

*SUT expression was consistent with genotype*

Increasing sucrose transporter expression has been suggested as a method of increasing photosynthesis and yield in crops (Ainsworth & Bush 2011; Stitt 2013), but previous experiments have shown mixed results (Leggewie *et al.* 2003; Dasgupta *et al.* 2014; Wang *et al.* 2015). In *Arabidopsis*, there was a decrease in biomass with increased sucrose transporter expression due to a perceived phosphate limitation (Dasgupta *et al.* 2014). No previous studies, however, have examined plants with greater sucrose transporter expression or activity at elevated [CO₂], which may heighten the potential benefit from increased phloem loading due to a greater potential for backup of leaf sugars at elevated [CO₂]. Two transgenic lines of *Arabidopsis thaliana* were tested in this experiment: one overexpressing AtSUC1 using the companion cell-specific viral promoter CoYMV, previously described in Dasgupta *et al.* (2014), and one replacing the native AtSUC2 with the barley sucrose transporter HvSUT1 driven by the AtSUC2 promoter.

Expression of AtSUC2, AtSUC1, and HvSUT1 in source leaves was confirmed using qRT-PCR. The AtSUC2 gene was knocked out in the HvSUT1 line, and there was virtually no expression of the gene in those plants (Fig. 5.1). Expression of the AtSUC2 gene was decreased in AtSUC1 plants, in contrast to results previously published by Dasgupta *et al.* (2014) on these lines. The AtSUC1 gene was expressed in all genotypes, but expression was greatly enhanced in the AtSUC1 line as expected (Table 5.2; Fig. 5.1). Native AtSUC1 is not associated with phloem loading, and is usually expressed in pollen, roots, and trichomes (Sivitz *et al.* 2007). In the AtSUC1 line, this native expression was enhanced by expression in the companion cells of phloem tissue driven by the CoYMV promoter. Although AtSUC2 expression was decreased in the AtSUC1 overexpressing genotype, overall expression of sucrose transporter genes increased
in this genotype (Fig. 5.1). In contrast, the HvSUT1 expressing genotype had lower overall sucrose transporter expression compared to wild-type (Fig. 5.1).

**AtSUC1 plants grow more slowly, particularly at ambient [CO₂] than HvSUT1 or wild-type plants**

Plants overexpressing AtSUC1 grew more slowly than wild-type plants or plants expressing HvSUT1, as indicated by rosette diameter (Fig. 5.2). This difference in growth, however, was less at elevated [CO₂]. A decrease in productivity was also observed by Dasgupta *et al.* (2014) in the AtSUC1 line at ambient [CO₂]. A similarity in size between HvSUT1 and wild-type plants has been observed by Reinders *et al.* (2012) at ambient [CO₂], as was observed in this experiment (Fig. 5.2). Although the HvSUT1 plants had overall lower expression of sucrose transporters (Fig. 5.1), the HvSUT1 transporter was able to still export sufficient sucrose to produce a healthy plant. This may be due to the HvSUT1 transporter having a higher activity and specificity than the native AtSUC2 transporter (Sivitz *et al.* 2005). The pattern of growth in rosette diameter corresponded well with visible leaf area (Table 5.3; Fig. 5.3d). Wild-type plants had a 27% stimulation in visible leaf area at elevated [CO₂], while HvSUT1 plants had a 1% stimulation and AtSUC1 plants had a 110% stimulation (Fig. 5.3d). Although AtSUC1 plants had much larger stimulation than either of the other genotypes, the leaf area was still lower than wild-type or HvSUT1 at elevated [CO₂]. A previous study also showed that AtSUC1 showed reductions in growth at ambient [CO₂], although these experiments were done at a lower light intensity (150 PPFD) and after a shorter period of growth (Dasgupta *et al.* 2014).

Total biomass was assessed at a similar developmental stage for each of the genotypes, with wild-type and HvSUT1 plants harvested four to five days before AtSUC1 plants. Wild-type and HvSUT1 plants were not significantly different in size (Fig 5.3) at ambient or elevated [CO₂]. Although leaf weight significantly increased in wild-type plants at elevated [CO₂], root weight did not change and HvSUT1 plants had no significant stimulation in any biomass parameters at elevated [CO₂] (Fig. 5.3). The AtSUC1 plants, however, had a very large stimulation in both leaf and root weight at elevated [CO₂], leading to a significant [CO₂] x genotype effect (Table 5.3). The difference in stimulation between wild-type and AtSUC1 plants was much greater in root weight (18% for wild-type plants, 124% for AtSUC1 plants) than in leaf weight (30% for wild-type plants, 61% for AtSUC1 plants).
The stimulation in growth and biomass at elevated [CO₂] in AtSUC1 plants was puzzling given that Dasgupta et al. (2014) had related a decrease in biomass at ambient [CO₂] to a perceived phosphate limitation. Plants at elevated [CO₂] generally take up more phosphate (Jin et al. 2015), so it would be expected that if the decrease in biomass was due to phosphate limitation, it would be exacerbated at elevated [CO₂]. The plants in this study, however, were fertilized throughout the experiment, so should have had ample phosphate. Dasgupta et al. (2014) observed that seedlings overexpressing sucrose transporters were able to return to normal size when grown on plates with increased phosphate. Perhaps the high phosphate in this experiment could have allowed the benefit from increased sucrose transporter expression to be enhanced at elevated [CO₂].

Changes in photosynthetic parameters and carbohydrate content in SUC/SUT lines at elevated [CO₂]

To determine whether the smaller size of AtSUC1 plants was related to photosynthesis, A/ci curves were taken to measure photosynthetic capacity. Dasgupta et al. (2014) observed a decrease in Fv/Fm in the AtSUC1 genotype, but did not measure photosynthetic CO₂ assimilation directly. There was a significant [CO₂] x genotype effect for maximum carboxylation rate (Vc,max) and maximum electron transport rate (Jmax), but no significant [CO₂] or genotype effect (Table 5.4; Fig. 5.4). There was a decrease in Vc,max at elevated [CO₂] in wild-type plants with no significant change in HvSUT1 or AtSUC1 plants (Fig. 5.4a) and an increase in Jmax in HvSUT1 plants at elevated [CO₂] with no significant change in Jmax in wild-type and AtSUC1 plants (Fig. 5.4b). The increase in Jmax in HvSUT1 at elevated [CO₂] did not translate to a stimulation in biomass (Fig. 5.3). In HvSUT1 and wild-type plants, there was a significant increase in the transition point between Rubisco- and electron transport-limited photosynthesis, while there was no significant change in AtSUC1 plants (Table 5.4; Fig. 5.4).

The similar photosynthetic capacity between AtSUC1 and wild-type plants was counter to Dasgupta et al. (2014)’s observation of a decrease in Fv/Fm in this genotype relative to wild-type. The plants in this experiment were grown under higher light and shorter day conditions than in the previous study. The A/ci measurements were also taken on a single fully expanded leaf, rather than over the entire rosette.
Despite the differences in sucrose transporter expression and type, there was no significant difference in leaf sucrose in either of the transgenic lines (Table 5.4; Fig. 5.5). There was a marginally significant decrease in hexose sugars (glucose + fructose) in AtSUC1 plants at both ambient and elevated [CO₂] (Fig. 5.5a). This was in contrast to Dasgupta et al. (2014)’s results, which found an increase in glucose, fructose, and sucrose in leaf tissue in AtSUC1 plants. In that study, however, they sampled whole rosettes, rather than individual source leaves. Therefore, if soluble sugars were accumulating in developing leaves, which act as sinks, they may mask any changes in soluble sugars in source leaves. There was an increase in sucrose content at elevated [CO₂] when averaged across genotypes (Fig. 5.5b).

Starch content was similar across all genotypes at ambient [CO₂], but increased substantially in wild-type and HvSUT1 plants at elevated [CO₂], while remaining low in AtSUC1 plants (Table 5.3; Fig. 5.5c). Similarly, specific leaf area (SLA) significantly decreased in wild-type and HvSUT1 plants exposed to elevated [CO₂], but did not change in AtSUC1 plants (Table 5.4; Fig. 5.5d). Dasgupta et al. (2014) observed no differences in starch content between the AtSUC1 and wild-type genotypes. The lower starch content in the AtSUC1 genotype compared to the other genotypes at elevated [CO₂] could be due to an increase in phloem loading in AtSUC1 plants (Dasgupta et al. 2014). Low starch content has been related to increased biomass in Arabidopsis (Sulpice et al. 2009) and the change in carbon dynamics in the leaf at elevated [CO₂] may indicate that more carbon was being used to stimulate growth in roots, since the relative increase in root biomass at elevated [CO₂] was much greater than the increase in leaf biomass in AtSUC1 plants (Fig. 5.3). The change in starch at elevated [CO₂] in HvSUT1 plants did not directly impact photosynthetic capacity (Fig. 5.4). Previous studies have observed increases in starch at elevated [CO₂] with little to no decrease in photosynthetic capacity (Ainsworth et al. 2004; Rogers et al. 2004; Davey et al. 2006; DaMattta et al. 2016), although in the wild-type Arabidopsis measured in this study, V_{c,max} was significantly lower at elevated [CO₂] and leaf starch content was more than doubled. Taken together, these results do not provide clear evidence for or against hypotheses about sugar-mediated feedback on photosynthesis at elevated [CO₂], and further studies would be needed to dissect the mechanisms of sugar-crosstalk in these transgenic lines.
No significant increase in phosphate limitation genes in AtSUC1 plants

Dasgupta et al. (2014) suggested that the decrease in growth in SUT overexpressing plants was due to a perceived phosphate limitation. The same genes, the purple acid phosphatases PAP14 and PAP24 and the inorganic phosphate transporters PHT2 and PT2, were tested in this study to determine whether the decrease in biomass in the AtSUC1 plants was consistent with increased perception of phosphate limitation (Table 5.2). Contrary to what was observed by Dasgupta et al. (2014), expression of these genes was not increased in AtSUC1 plants and often marginally decreased (Table 5.2; Fig. 5.6). In wild-type plants, the expression of PAP14, PAP24, and PT2 significantly increased at elevated $[CO_2]$, while the expression did not significantly change in either the HvSUT1 or the AtSUC1 plants. The $[CO_2] \times$ genotype interaction, however, was not significant. There was no significant effect of $[CO_2]$ on PHT2 expression.

The increase in phosphate limitation genes in wild-type at elevated $[CO_2]$ was contrary to previous microarray experiments, where expression of PT2 increased slightly at elevated $[CO_2]$, but none of the other genes changed in fully developed leaves (Markelz et al. 2014a, b). In a study of Arabidopsis grown hydroponically, however, many phosphate transport related genes, such as the phosphate transporter PHT1 and the purple acid phosphatase PAP2, were up-regulated at elevated $[CO_2]$ (Niu et al. 2012). Similarly, the activity of acid phosphatases in the soil of a poplar stand increased at elevated $[CO_2]$ (Lagomarsino et al. 2008). PHT2 did not increase at elevated $[CO_2]$, but PHT2 expression in leaves is often not tied to phosphate limitation (Daram et al. 1999; Hammond et al. 2003).

Although the AtSUC1 genotype was much smaller at elevated $[CO_2]$, the decrease in size was not tied to an increase in phosphate limitation genes as found by Dasgupta et al. (2014). Wang et al. (2015) also observed increases in the expression of two phosphate transporters in the developing seeds of transgenic rice overexpressing sucrose transporters, along with a general increase in expression of genes related to nutrient remobilization. When they overexpressed AtSUC2 in rice, they found an increase in biomass and yield, rather than a decrease. Many of the genes analyzed by Dasgupta et al. (2014) and in this experiment were expressed weakly in leaf tissue (Muchhal et al. 1996; Zhu et al. 2005; Jost et al. 2015; Fig. 5.6), and so changes in expression may be difficult to detect. The plants in this experiment were fertilized often, which may decrease the effects of perceived phosphate limitation (Dasgupta et al. 2014). Further studies will be needed to determine why the AtSUC1 plants were smaller than wild-type, and
why the AtSUC1 plants had a much greater stimulation in biomass than wild-type at elevated [CO₂].

Limitations of This Study

Although it was assumed that greater SUT expression in the transgenic lines would result in greater SUT activity, SUT protein content and activity were not directly measured, and would need to be in order to be certain that the transcriptional changes impacted function. SUTs generally have a high turnover rate (Kuhn et al. 1997; Vaughn et al. 2002) and in beet, the expression of BvSUT1, a closely related SUT used for phloem loading, was directly tied to sucrose transport activity (Vaughn et al. 2002), but the connection was not directly tested in this experiment or in AtSUC2. Dasgupta et al. (2014) determined an increase in sucrose transport in the AtSUC1 line through C14 tracer experiments, but this was not confirmed in the current experiment nor was the sucrose transport activity determined the HvSUT1 line. Another limitation of this work was the focus on a single transgenic line for each transgenic event. Experimentation with additional lines may help with the interpretation of the results reported here.

Conclusions

This study investigated whether increasing sucrose transporter expression or activity could provide a greater benefit to A. thaliana plants from elevated [CO₂]. Previous experiments have found a decrease in biomass in Arabidopsis overexpressing AtSUC1, due to a perceived phosphate limitation. In this study, biomass was decreased in the AtSUC1 genotype, but not in the HvSUT1 genotype, an atsuc2 knock-out complimented with the barley sucrose transporter HvSUT1, compared to wild-type. Elevated [CO₂] stimulated biomass much more strongly in the AtSUC1 genotype than in HvSUT1 or wild-type, however. Genes related to phosphate limitation were not upregulated in the AtSUC1 genotype, unlike previous studies. Therefore, further experiments may be needed to determine why the AtSUC1 genotype had decreased biomass compared to wild-type and such a strong stimulation in biomass at elevated [CO₂].
### TABLES AND FIGURES

**Table 5.1.** Oligonucleotides used for qRT-PCR analysis of expression for indicated phosphate and sucrose transport genes.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP14 Forward</td>
<td>TGTGCGAGACAAAGTGACGTGG</td>
<td>Dasgupta et al., 2014</td>
</tr>
<tr>
<td>PAP14 Reverse</td>
<td>GATTCGATCGCAGGAGCAA</td>
<td></td>
</tr>
<tr>
<td>PAP24 Forward</td>
<td>CCACCAATGATTGGGTAGGCA</td>
<td>Dasgupta et al., 2014</td>
</tr>
<tr>
<td>PAP24 Reverse</td>
<td>AGGCTTTCTCTTTCCCATAGGCT</td>
<td></td>
</tr>
<tr>
<td>PHT2;1 Forward</td>
<td>CATTCTCCAAAACGGAGCAG</td>
<td>Dasgupta et al., 2014</td>
</tr>
<tr>
<td>PHT2;1 Reverse</td>
<td>CGAGAACATCCATTTGGGATAA</td>
<td></td>
</tr>
<tr>
<td>PT2 Forward</td>
<td>CGAAGCTCCTCGGTGCTAT</td>
<td>Dasgupta et al., 2014</td>
</tr>
<tr>
<td>PT2 Reverse</td>
<td>GGAGAGTCCAGGCTTTTGT</td>
<td></td>
</tr>
<tr>
<td>AtSUC1 Forward</td>
<td>GTCTCCTTTTCATCGCCACC</td>
<td>Dasgupta et al., 2014</td>
</tr>
<tr>
<td>AtSUC1 Reverse</td>
<td>TTGTTGGCTACGTCGAGGA</td>
<td></td>
</tr>
<tr>
<td>AtSUC2 Forward</td>
<td>TAGCCATTGTCGTCCCTCAGATG</td>
<td>Dasgupta et al., 2014</td>
</tr>
<tr>
<td>AtSUC2 Reverse</td>
<td>ATGAAATCCCATAGTACGCTTTGAAGG</td>
<td></td>
</tr>
<tr>
<td>HvSUT1 Forward</td>
<td>TCTTGGATTCTGGCTTTTGA</td>
<td>This study</td>
</tr>
<tr>
<td>HvSUT1 Reverse</td>
<td>GGAAACCATTTGTCGCCAGTA</td>
<td></td>
</tr>
<tr>
<td>qREF Forward</td>
<td>GAGCTGAAGTGGCTTCCATGAC</td>
<td>Czechowski et al., 2005</td>
</tr>
<tr>
<td>qREF Reverse</td>
<td>GGTCGACATACCATGATCC</td>
<td></td>
</tr>
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</table>
Table 5.2. Analysis of variance of the relative expression of genes across the three genotypes exposed to ambient and elevated \([\text{CO}_2]\). Samples for measurement of RNA expression were taken at 38 DAP for HvSUT1 and wild-type plants and at 43 DAP for AtSUC1 plants. Significant \((p < 0.05)\) effects are bolded, marginally significant \((p < 0.10)\) effects are italicized.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>([\text{CO}_2]); F, p</th>
<th>Genotype</th>
<th>([\text{CO}_2]) x Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUT2</td>
<td>2.45, 0.14</td>
<td></td>
<td>1020, (&lt;0.0001)</td>
</tr>
<tr>
<td>SUT1</td>
<td>1.12, 0.31</td>
<td></td>
<td>40.6, (&lt;0.0001)</td>
</tr>
<tr>
<td>HvSUT1</td>
<td>0.17, 0.69</td>
<td></td>
<td>23.3, (0.001)</td>
</tr>
<tr>
<td>PAP14</td>
<td>\textbf{10.6, 0.005}</td>
<td></td>
<td>3.70, (0.05)</td>
</tr>
<tr>
<td>PAP24</td>
<td>\textbf{7.51, 0.01}</td>
<td></td>
<td>3.04, (0.07)</td>
</tr>
<tr>
<td>PHT2</td>
<td>1.48, 0.24</td>
<td></td>
<td>2.69, (0.10)</td>
</tr>
<tr>
<td>PT2</td>
<td>\textbf{6.67, 0.02}</td>
<td></td>
<td>2.53, (0.11)</td>
</tr>
</tbody>
</table>
Table 5.3. Analysis of variance of biomass and leaf count / area parameters across the three genotypes exposed to ambient and elevated [CO$_2$]. Leaf area was taken at 32 DAP and harvest was taken at 39-40 DAP for wild-type and HvSUT1 plants and at 44 DAP for AtSUC1 plants. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>[CO$_2$]: F, P</th>
<th>Genotype</th>
<th>[CO$_2$] x Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Count</td>
<td>1.33, 0.25</td>
<td><strong>8.21, 0.0005</strong></td>
<td><strong>3.39, 0.04</strong></td>
</tr>
<tr>
<td>Leaf Weight</td>
<td><strong>14.3, 0.0003</strong></td>
<td>4.23, 0.02</td>
<td>1.61, 0.21</td>
</tr>
<tr>
<td>Root Weight</td>
<td><strong>9.81, 0.002</strong></td>
<td><strong>7.79, 0.0008</strong></td>
<td><strong>8.16, 0.0006</strong></td>
</tr>
<tr>
<td>Flower Weight</td>
<td>0.41, 0.53</td>
<td>2.66, 0.08</td>
<td>1.05, 0.36</td>
</tr>
<tr>
<td>Total Weight</td>
<td><strong>7.96, 0.006</strong></td>
<td><strong>4.37, 0.02</strong></td>
<td><strong>3.62, 0.03</strong></td>
</tr>
<tr>
<td>Leaf Area</td>
<td><strong>10.8, 0.002</strong></td>
<td><strong>40.3, &lt;0.0001</strong></td>
<td><strong>2.43, 0.09</strong></td>
</tr>
</tbody>
</table>
Table 5.4. Analysis of variance of photosynthetic parameters taken from A/ci curves and leaf carbohydrate measurements across the three genotypes exposed to ambient and elevated [CO₂]. Photosynthesis measurements were performed at 37 DAP for HvSUT1 and wild-type plants and at 42 DAP for AtSUC1 plants. Samples for carbohydrate content were taken at 38 DAP for HvSUT1 and wild-type plants and at 43 DAP for AtSUC1 plants. Significant (p < 0.05) effects are bolded, marginally significant (p < 0.10) effects are italicized.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>[CO₂]: F, P</th>
<th>Genotype</th>
<th>[CO₂] x Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vél, max</td>
<td>0.78, 0.38</td>
<td>0.62, 0.55</td>
<td>3.63, 0.04</td>
</tr>
<tr>
<td>Jmax</td>
<td>1.20, 0.28</td>
<td>1.00, 0.38</td>
<td>3.89, 0.03</td>
</tr>
<tr>
<td>Transition Point</td>
<td>14.5, 0.0009</td>
<td>2.82, 0.08</td>
<td>10.6, 0.0005</td>
</tr>
<tr>
<td>Hexose</td>
<td>3.02, 0.09</td>
<td>2.88, 0.07</td>
<td>0.33, 0.72</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.13, 0.004</td>
<td>0.99, 0.38</td>
<td>1.17, 0.32</td>
</tr>
<tr>
<td>Starch</td>
<td>60.2, &lt;0.0001</td>
<td>13.1, &lt;0.0001</td>
<td>5.44, 0.008</td>
</tr>
<tr>
<td>SLA</td>
<td>19.7, &lt;0.0001</td>
<td>1.46, 0.24</td>
<td>1.83, 0.17</td>
</tr>
</tbody>
</table>
Figure 5.1. Relative expression, as compared to a reference gene, of the sucrose transporters AtSUC1 (solid bars), AtSUC2 (increasing sloped bars), and HvSUT1 (decreasing sloped bars). Samples were taken at 38 DAP for HvSUT1 and wild-type plants and at 43 DAP for AtSUC1 plants. Means are separated using LSD, with different letters indicating significantly different mean values. Each symbol represents the mean (± standard error) of four samples, with leaves from two plants used for each sample.
Figure 5.2. Diameter of the rosette, measured every 3-4 days from emergence until just before harvest of the wildtype and HvSUT1 plants. Each symbol represents the mean (± standard error) of ten plants, except wildtype ambient [CO₂], which represents the mean of nine plants.
Figure 5.3. The total biomass (a), final leaf number (b), leaf weight (c), visible leaf area (d), root weight (e), and flower weight (f) in ambient [CO₂] (white bars) or elevated [CO₂] (black bars) grown plants. Leaf area was taken at 32 DAP and harvest was taken at 39-40 DAP for wild-type and HvSUT1 plants and at 44 DAP for AtSUC1 plants. Means are separated using LSD, with different letters indicating significantly different mean values. Each symbol represents the mean (± standard error) of 14-18 plants.
Figure 5.4. The maximum carboxylation rate ($V_{c,max}$; a), maximum electron transport rate ($J_{max}$; b), and the $c_i$ value at the transition point between Rubisco limited and electron transport limited photosynthesis in the $A/c_i$ curve (c) in ambient [CO$_2$] (white bars) or elevated [CO$_2$] (black bars) grown plants. Photosynthesis measurements were performed at 37 DAP for HvSUT1 and wild-type plants and at 42 DAP for AtSUC1 plants. Means are separated using LSD, with different letters indicating significantly different mean values. Each symbol represents the mean ($\pm$ standard error) of five plants.
Figure 5.5. Leaf hexose (glucose + fructose; a), sucrose (b), and starch (c) content and specific leaf area (d) in ambient [CO₂] (white bars) or elevated [CO₂] (black bars) grown plants. Samples were taken at 38 DAP for HvSUT1 and wild-type plants and at 43 DAP for AtSUC1 plants. Means are separated using LSD, with different letters indicating significantly different mean values. Each symbol represents the mean (± standard error) of eight plants, except starch in the AtSUC1 ambient [CO₂] plants and specific leaf area in the wild-type ambient [CO₂] plants, which both represent the mean of seven plants.
Figure 5.6. Relative expression, as compared to a reference gene, of the purple acid phosphatases PAP14 (a) and PAP24 (b), and the inorganic phosphate transporters PHT2 (c) and PT2 (d). Samples were taken at 38 DAP for HvSUT1 and wild-type plants and at 43 DAP for AtSUC1 plants. Means are separated using LSD, with different letters indicating significantly different mean values. Each symbol represents the mean (± standard error) of four samples, with leaves from two plants used for each sample.
CHAPTER VI: CONCLUDING REMARKS

Current projections show that atmospheric carbon dioxide concentration ([CO₂]) will continue to increase in the next century and is expected to exceed 500 ppm by 2050 (Ciais et al. 2013). Elevated [CO₂] directly increases C₃ photosynthesis by raising [CO₂] at the site of Rubisco and consequently increasing the velocity of carboxylation and decreasing photorespiration (Ainsworth & Long 2005; Leakey et al. 2009a). The increase in photosynthesis typically results in greater biomass production and crop yields at elevated [CO₂] (Ainsworth & Long 2005). However, the benefit from elevated [CO₂] can vary depending on environment (Kimball et al. 1995; Ruiz-Vera et al. 2013), plant functional type (Ainsworth & Long 2005), genotype or cultivar within a species (Hasegawa et al. 2013), and sink strength (Ainsworth et al. 2004; Aranjuelo et al. 2013). This variation is apparent in a meta-analysis of crop responses to elevated [CO₂], where for each species, the 95% confidence intervals surrounding the mean response of photosynthesis, biomass production or seed yield to elevated [CO₂] are large (Fig. 2.1). This thesis research aimed to test the drivers of these large confidence intervals in order to better understand crop responses to elevated [CO₂] which would aid in improving the accuracy of modeling efforts, and crop breeding applications.

Overall, these studies demonstrated that there is significant variation in plant responses to elevated [CO₂], driven both by environmental and intraspecific differences. In times and locations when there was less water available, plants responded less to elevated [CO₂] in a meta-analysis of FACE and OTC crop experiments (Chapter 2). Sink strength was also an important determinant of soybean yield response to elevated [CO₂] (Chapter 3), and plants with different phloem loading strategies did not have fundamentally different responses to elevated [CO₂] (Chapter 4). Only four species were measured, but the anatomical, biochemical and physiological data do not support a strong role for phloem loading strategy in determining photosynthetic response to elevated [CO₂]. Similarly, transgenic effects to increase expression of sucrose transporters did not increase biomass or photosynthesis at elevated [CO₂] (Chapter 5).

This research provided important insights that can be used for better estimating and maximizing response to elevated [CO₂]. Taking the variation in [CO₂] response with changes in environment, species, or cultivar into account is critical for future modeling efforts, which may over- or underestimate the impact of [CO₂] on crops otherwise. Demonstrating significant variation in [CO₂] response in soybean is an important first step in breeding soybeans for greater
yield at elevated [CO₂] (Ainsworth et al. 2008). The results on the relationship between phloem loading and [CO₂] response could provide insights as to how source: sink dynamics impact [CO₂] response. The results also provide a basis for many future experiments. More studies on the response of crops to elevated [CO₂] in tropical locations are necessary to determine if the relationship between environment and [CO₂] response found in Chapter 2 holds true in other locations. In addition, the variation in soybean yield response to elevated [CO₂] could be used to produce recombinant inbred lines or for further physiological studies of genotypes such as HS93-4118 and Loda which have very different responses to elevated [CO₂]. Additional species with different phloem loading strategies, including polymer trapping species, could be studied to further determine whether there is still no impact of phloem loading strategy on response to elevated [CO₂] as observed in Chapter 4 and further passive and apoplastic loading species should be tested to determine if the differences in the diurnal progression of carbohydrates and transition point between plants with apoplastic or passive loading hold true across a wider range of species. Chapter 5 was a preliminary study, and so further experimentation will be needed to tease out the relationship between expression of sucrose transporters and response to elevated [CO₂]. Determining the exact transport capacity as well as testing other lines of the same constructs would be necessary. Testing the relationship between phloem loading capacity and [CO₂] response in other species, particularly those with large reproductive sinks would provide further insight into this relationship as well. This research in total determined that both environment and cultivar play a role in response to elevated [CO₂], while phloem loading strategy does not and enhancing phloem loading expression did not increase photosynthesis or biomass at elevated [CO₂].
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APPENDIX A: SOURCES OF DATA USED IN THE META-ANALYSIS FOR CHAPTER II.

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APPENDIX B: SOURCES OF DATA USED IN THE META-ANALYSIS FOR CHAPTER IV.


