Genetic Variation in Photosynthesis as a Tool for Finding Principal Routes to Enhancing Photosynthetic Efficiency

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Nicholas J. Tomeo
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by

NICHOLAS J. TOMEO

has been approved for
the Department of Environmental and Plant Biology
and the College of Arts and Sciences by

David M. Rosenthal
Assistant Professor of Environmental and Plant Biology

Robert Frank
Dean, College of Arts and Sciences
Abstract

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Genetic Variation in Photosynthesis as a Tool for Finding Principal Routes to Enhancing Photosynthetic Efficiency

Director of Dissertation: David M. Rosenthal

Throughout this dissertation I approach the long-term aim of improving photosynthesis through the lens of natural genetic variation for photosynthesis. To date few studies have directly asked how photosynthetic variation might inform or provide the genetic material required to enhance photosynthesis, despite the clear utility of this strategy for other types of agronomic improvement. Of the many traits underlying variation in photosynthesis, mesophyll conductance – the diffusional flux of CO₂ through the leaf interior – has potential to improve both photosynthesis and water use efficiency. I assess genetic variation for photosynthesis among ecotypes of the model plant Arabidopsis thaliana and cultivars of soybean (Glycine max). In both species, and across both controlled and field environments in soybean, I find heritable genetic variation for mesophyll conductance that is positively correlated to variation in photosynthetic rate, indicating that selection to enhance mesophyll conductance will increase photosynthesis. Genetic variation in mesophyll conductance though was largely unrelated to variance in water use efficiency as a result of phenotypic correlation between stomatal and mesophyll conductance. If trait variation is to prove useful for crop breeding, that trait must not have already been improved in the varieties currently used by farmers. In soybean, photosynthesis has improved slightly with breeding for yield across a historical set of cultivars. Mesophyll conductance is not responsible for this increase in photosynthesis; it remains unchanged after 75 years of selection for yield.
Stomatal conductance is greater in modern varieties and I show that this increase scales from the leaf to the canopy. Greater canopy conductance in modern soybeans resulted in lower canopy temperatures and reduced leaf heat-stress. Few leaf-level photosynthetic traits were improved across this historical set of soybean cultivars. Given that I observed heritable genetic variation for mesophyll conductance among a small sampling of available soybean germplasm, there is substantial opportunity to harness this variation for the improvement of photosynthetic efficiency.
Dedication

For her unwavering support and motivation,

this work is dedicated to my wife Tiffany.
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Chapter 1. Background Material and Introduction to Proceeding Chapters

Background

Ending hunger is the second of the seventeen United Nations Goals of Sustainable Development (2017). Despite steady reductions in global hunger over the past 20 years, one-in-nine still do not have enough to eat (FAO, 2013). Growing prosperity in developing nations is causing diets to become more calorie-intensive (Keyzer et al., 2005), and in developed nations the emergence of biofuels markets (Balat and Balat, 2009) has created demand for feedstocks. The demand for feed and fuel will continue to rise as world population grows by two billion over the next four decades (Population Data Sheet, 2013). In order to alleviate food insecurity around the world, feed all of the planet’s new people, and ensure continued economic stability we must pursue efforts to increase crop productivity (Edgerton, 2009; Godfray et al., 2010).

Yield growth of staple crops has slowed over the past twenty years (Alston et al., 2009) as the components of yield potential historically responsible for yield increases approach their theoretical upper limits (Zhu et al., 2010). Crop yield potential (i.e., the expected yield of a genotype under ideal conditions; \( Y = S_t \ast \epsilon_i \ast \epsilon_c \ast \epsilon_p \)) is a function of the quantity of photosynthetically active radiation incident upon a field throughout the growing season (\( S_t \)), the efficiency of light interception by the canopy (\( \epsilon_i \)), the conversion efficiency of absorbed light to biomass (\( \epsilon_c \)) and the proportion of biomass partitioned to useable product (\( \epsilon_p \)) (Monteith, 1977; Slattery et al., 2013). Gains in yields over the past century are primarily attributable to the development of cultivars with greater \( \epsilon_p \) (Hay, 1995; Koester et al., 2014), and faster more complete canopy development leading to higher \( \epsilon_i \) (Zhu et al., 2010; Koester et al., 2014). Conversion efficiency has received little attention with regards to crop improvement. Conversion efficiency is determined in part
by the photosynthetic rate of all leaves in a canopy less respiration, thus all other things equal, any increase in photosynthetic rates should improve yields (Long et al., 2006). Routes to increasing crop photosynthetic rates with the intent of raising conversion efficiency and improving yields are extensively reviewed in the literature (Long et al., 2006; Zhu et al., 2008; von Caemmerer and Evans, 2010; Zhu et al., 2010; Ainsworth et al., 2012; Flexas et al., 2013; Singh et al., 2014; Ort et al., 2015; Slattery and Ort, 2015; Flexas et al., 2016; Kromdijk et al., 2016; Niinemets et al., 2017). Some strategies propose to re-engineering leaf biochemistry through long-term bioengineering projects such as altering the carbon-fixing enzyme Rubisco or implementing CO₂-concentrating mechanisms in C₃ crops (Long et al., 2006; von Caemmerer et al., 2012), while other strategies rely on simpler traditional recombinant DNA technologies to increase or alter endogenous gene expression (e.g., up regulating rate-limiting enzymes of the Calvin cycle) (Tamoi et al., 2006; Raines, 2011; Evans, 2013). Alternatively, reducing the cost associated with photorespiration is being pursued by several research groups (Kebeish et al., 2007; Maurino and Függe 2009; Ort et al., 2015). Photorespiration is an energetically costly pathway that effectively scavenges previously fixed carbon after oxygenation events, but substantially reduces net photosynthesis (Peterhansel and Maurino, 2011). Most of these strategies are mutually beneficial and we will achieve the greatest enhancements to photosynthetic efficiency through the combination of strategies.

Throughout this dissertation I approach the long-term aim of improving photosynthesis through the lens of natural genetic variation for photosynthesis. In this context I am interested in genetic variation defined as uncharacterized differences among genotypes that lead to consistent, differentiable phenotypes or quantitative traits.
There is standing genetic variation for photosynthetic physiology at multiple scales of biological organization (i.e., across plant families and within species) and this variation provides a powerful tool to further characterize the mechanistic and genetic basis of important traits. To date few studies have directly asked how photosynthetic variation might inform or provide the genetic material required to enhance photosynthesis (Flood et al., 2011), despite the clear utility of this strategy for other types of agronomic improvement. For example, selection on natural genetic variation has enhanced yields by providing resistance to pests and pathogens (Foster et al., 1991; Hill et al., 2006), improving heat and drought tolerance (Maestri et al., 2002; Rebetzke et al., 2002), increasing harvest indices (Hay, 1995), and supplying the “genes of the Green Revolution” – the dwarfing mutations in wheat and indica rice (Hedden, 2003). Given that photosynthetic physiology varies extensively among both populations of wild species (Geber and Dawson, 1997; Brouillette et al., 2014; Easlon et al., 2014; Momayyezi and Guy, 2017) and cultivars of crops (Barbour et al., 2010; Giuliani et al., 2013; Gu et al., 2014b; Barbour et al., 2016), it stands to reason that this variation may be useful to increasing yields as well. Of the many traits underling variation in photosynthesis, the rate of CO₂ diffusion through leaves has potential to improve both photosynthesis and water use efficiency (Zhu et al., 2010; Flexas et al., 2013; Flexas et al., 2016).

The photosynthetic assimilation of CO₂ into organic molecules occurs within the fluid matrix of the chloroplasts. In order for CO₂ to reach the chloroplast interior it must pass through several internal barriers (Figure 1.1)(Nobel, 2005). Each of these barriers potentially limits the transfer of CO₂ to the chloroplast – where Ribulose 1,5-bosphosphate carboxylase oxygenase (Rubisco) catalyzes the fixation of CO₂ into organic form –
effectively limiting supply of the substrate CO$_2$ to Rubisco, slowing catalysis and ultimately photosynthesis (Flexas et al., 2008; Tholen and Zhu, 2011). The CO$_2$ concentration is first reduced as it diffuses through the stomatal pores on the leaf surface to the intercellular air spaces, a process referred to as stomatal conductance ($g_s$). Barriers from leaf intercellular air spaces to the chloroplast are collectively referred to as mesophyll conductance ($g_m$). Both $g_s$ and $g_m$ can significantly limit photosynthetic rates, with each component typically constituting 20-50% of the total limitation to net photosynthesis (Grassi and Magnani, 2005; Perez-Martin et al., 2009; Tomás et al., 2013). Increasing $g_m$ would have the effect of reducing the total diffusional limitation, increasing the rate of carboxylation, and thus photosynthesis.

The extent that genetic variation exists for $g_m$ and how that variation relates to photosynthesis intraspecifically is not well characterized. Mesophyll conductance varies drastically across taxa (Flexas et al., 2008), for example, at least 24-fold in seed plants (Tomás et al., 2013), providing motivation for the hypothesis that $g_m$ ought to vary across crop cultivars or genotypes of wild plants as well (Barbour et al., 2010; Giuliani et al., 2013; Jahan et al., 2014). Mesophyll conductance should also influence leaf-level intrinsic water use efficiency ($WUE$, defined here as moles of CO$_2$ assimilated per mole of water lost: $WUE = \frac{\text{net photosynthetic rate}}{g_s}$) since any increase in $g_m$ should increase photosynthesis, but be independent of water loss from the leaf. Higher $g_m$ increases the CO$_2$ concentration around Rubisco which increases carboxylation rates without directly altering $g_s$, and thus leading to greater $WUE$ (Flexas et al., 2012). In agricultural contexts $g_m$ is of interest because cultivars with the greatest $g_m$ could offer the raw genetic material for breeding crops with greater $WUE$ and yields under water-limiting conditions (Flexas et al., 2012; Adachi et al., 2013), an important
long-term goal for closing yield gaps in sub-optimal environments (Flexas et al., 2013). In an ecological context WUE can directly impact plant survival and geographic range (Geber and Dawson, 1997; Geber and Griffen, 2003). Intraspecific variation in $g_m$ therefore, would be ecologically and evolutionarily meaningful through influencing plant distributions and fitness.

To date only a handful of studies have attempted to quantify variation in $g_m$ across wild plant genotypes or crop cultivars. Among eight tomato cultivars little variation in $g_m$ was observed (Galmés et al., 2011), while across wild tomato species in the genus Solanum approximately two-fold variation was observed (Muir et al., 2014; Muir et al., 2017). Similarly, in rice four cultivars exhibited modest variation, while a greater range was seen across wild relatives (Giuliani et al., 2013). Finally, among small selections of barley (Barbour et al., 2010) and wheat cultivars (Jahan et al., 2014) $g_m$ varied two-fold. In all of these studies net-photosynthesis was positively correlated with $g_m$, and together they suggest using natural genetic variation in $g_m$ as a mechanism to increase photosynthesis, WUE, and yields of crops is a plausible strategy.

Efforts to investigate mesophyll conductance in crops have focused primarily on cereals, specifically wheat (Jahan et al., 2014; Barbour et al., 2016), rice (Adachi et al., 2013; Giuliani et al., 2013; Gu et al., 2014a), and barley (Barbour et al., 2010). Of eudicot species, intraspecific variation in $g_m$ has only been studied in grape (Tomás et al., 2014) and tomato (Galmés et al., 2011). In 2014 (the latest year that data is available), half of the top ten most produced crops globally were eudicots (FAOSTAT 2017; http://www.fao.org/faostat/en/#data/QC), none of which were grape or tomato. Soybean production was fourth globally, and was sixth in terms of total land area planted.
Yields of soybean have improved drastically over the past century; in U.S. varieties yields increased 23 kg ha\(^{-1}\) year\(^{-1}\) between 1923 and 2008 (Rincker et al., 2014). Interception and partitioning efficiencies in modern soybean varieties are near their theoretical optima (Zhu et al., 2010; Koester et al., 2014) and further yield improvements are largely contingent upon increasing photosynthetic efficiency (Ainsworth et al., 2012). Given its economic importance and the necessity of enhancing photosynthesis to improve yields, soybean is an ideal model for investigating genetic variation in mesophyll conductance. Conversion efficiency in soybean has increased through historical yield improvements (Koester et al., 2014), and improvements in photosynthesis, though generally modest, have now been reported in several studies with unique historical sets of cultivars (Morrison et al., 1999; Jin et al., 2010; Cui et al., 2016; Koester et al., 2016; Li et al., 2017). Koester et al., (2016) recently observed that over 80 years of improvements in U.S. soybeans, mesophyll conductance was unchanged (i.e., modern and historical cultivars had equivalent \(g_m\)), indicating there is still potential to improve this trait. Coincident with enhanced photosynthesis, greater stomatal conductance is consistently observed in modern soybean cultivars (Morrison et al., 1999; Jin et al., 2010; Cui et al., 2016; Koester et al., 2016; Li et al., 2017), a common corollary with yield enhancement among many C\(_3\) crops (Roche, 2015). That greater photosynthesis has historically resulted from reductions in total diffusional resistance to CO\(_2\) into leaves by means of elevated stomatal conductance provides empirical evidence that greater mesophyll conductance would deliver the same result.

For any trait to be useful for selection in a breeding program it must meet several criteria. The trait should (1) vary across cultivars with a large enough magnitude to warrant selection, (2) be sufficiently heritable to allow for selection, and (3) have not
been selected through past cultivar development such that modern cultivars already exhibit the greatest values of the trait (Richards, 1996; Araus, 2002). We know in U.S. soybean that mesophyll conductance has not yet been selected (Koester et al., 2016). Genetic variation for mesophyll conductance seems apparent in some crop species and in some cases the variance among cultivars is of meaningful magnitude (Barbour et al., 2010; Adachi et al., 2013; Giuliani et al., 2013; Tomás et al., 2014; Barbour et al., 2016), though estimates of heritability for $g_m$ are lacking in both crops and natural plant populations.

**Introduction to chapters**

The following chapters will investigate a number of questions and hypotheses related to genetic variation in photosynthetic trait physiology and how trait variation might inform strategies to enhance photosynthetic rates. Chapter 2 looks broadly at the quantitative genetics of photosynthetic traits in natural populations of the model plant *Arabidopsis thaliana*. Several specific questions are addressed: (i) which traits are heritable, (ii) are there genetic correlations between traits, (iii) specifically is there heritable genetic variation for mesophyll conductance and does it correlate with photosynthetic rates or water-use-efficiency, and (iv) is there genetic variation for photorespiration and does it correlate with photosynthesis? In Chapter 3 we evaluate genetic variation for mesophyll conductance and its relationship to photosynthesis and water use efficiency in a diverse assortment of soybean cultivars. We begin by assessing this variation in a controlled environment, then test for variation and covariation of traits in a field environment, and assess the consistency of results from controlled to field environments. In Chapter 4 we survey photosynthetic trait variation in the historical set of soybean cultivars used by Koester et al., (2016), and ask if the relationship of increased photosynthesis resulting
from greater stomatal conductance in modern cultivars has had additional costs. We specifically question: when comparing modern vs. older cultivars if (i) greater leaf level stomatal conductance scales up to the canopy, (ii) greater conductance is facilitated by reduced water stress, (iii) greater conductance reduces canopy temperature, and (iv) lower canopy temperature reduces leaf heat stress? Together the studies presented here provide a comprehensive assessment of where natural genetic variation in photosynthesis may inform traditional and bioengineering approaches to enhancing C₃ eudicot crop photosynthesis, and explicitly in soybean demonstrate where future efforts might prove fruitful – and equally important where they will not.

References


bypass increases photosynthesis and biomass production in Arabidopsis thaliana.

Nat Biotechnol 25: 593-599


and scaling up by models. J Exp Bot 64: 2269–2281


Figure 1.1 Carbon dioxide must diffuse from the atmosphere ($C_a$) into the leaf through the stoma to the intercellular air space ($C_i$), then through the cell wall and plasma membrane to the cytoplasm of individual cells where it diffuses to and through the chloroplast envelope. Rubisco is solely housed in the chloroplast. Each step along the diffusion path resists the transport of CO$_2$ such that the concentration of CO$_2$ decreases from $C_a$ to $C_i$ to $C_c$. The CO$_2$ diffusion path is drawn in red: $C_a$ to $C_i$ represents the stomatal conductance to CO$_2$ ($g_{s-co2}$), while $C_i$ to $C_c$ represents mesophyll conductance to CO$_2$ ($g_m$). The interior airspaces of leaves are assumed saturated with water vapor ($W_i$) and that vapor diffuses out of the stoma toward the much drier ambient air ($W_a$).
Chapter 2. Natural Genetic Variation in Photosynthesis and Photorespiration

Among Arabidopsis thaliana Ecotypes

Abstract

Standing natural genetic variation in photosynthesis is recognized as a resource for explaining how plant populations have diverged, and yet is underutilized as a commodity for crop improvement. A greater understanding of natural variation in photosynthesis will provide insights for further refining strategies to crop improvement and may in the process demonstrate overlooked complications or opportunities. Using Arabidopsis thaliana ecotypes as a model system we ask if the drivers of photosynthesis are genetically correlated, if and how photosynthesis and photorespiration are physiologically coordinated, and how mesophyll conductance influences water use efficiency. We find standing genetic variation – as broad-sense heritability – for most photosynthetic traits, including photorespiration. Significant genetic correlation between photosynthetic electron transport and carboxylation capacities confirm that these traits are genetically constrained. Phenotypic variation in photorespiratory CO$_2$ efflux was correlated with both photosynthetic capacity (as maximum carboxylation and electron transport) and assimilation rates, though no genetic correlations were detected. Ecotypes with a winter annual life history habit had greater mesophyll conductance, maximum carboxylation capacity, maximum electron transport capacity, and leaf structural robustness. Stomatal conductance varied little in winter habit ecotypes, allowing for a positively correlation between integrated water use efficiency and mesophyll conductance, demonstrating that mesophyll conductance can modulate water use efficiency intraspecifically. Genetic correlations between traits supplying energy and carbon to the Calvin cycle suggest that independent selection on any one of these traits
will improve all of them. Likewise, it is appealing to interpret a lack genetic correlation between photosynthesis and photorespiration as an indication that positive scaling of these traits can be broken.

**Introduction**

Few studies have explicitly addressed how we might harness natural genetic variation in photosynthesis (Flood et al., 2011) even though improving photosynthetic efficiency will allow for continued crop yield enhancement (von Caemmerer and Evans, 2010; Zhu et al., 2010). Naturally occurring genetic variation is a useful tool for unraveling the mechanistic and genetic basis of both ecologically and agronomically important plant traits (Flood et al., 2011). From an ecological perspective natural genetic variation for traits across different populations is illustrative of the history of selection and can highlight differential selection pressures on those populations (Antonovics and Bradshaw, 1968; Geber and Dawson, 1990; Dudley, 1996; Franks et al., 2007). For example, a latitudinal gradient in freezing tolerance (Zhen and Ungerer, 2008) contributes to fitness advantages among *Arabidopsis thaliana* populations (Ågren and Schemske, 2012). In an agricultural context natural genetic variation has provided many yield enhancing traits including pathogen and pest resistance (Foster et al., 1991; Hill et al., 2006), improved heat tolerance (Maestri et al., 2002), and yield enhancement through increasing harvest indices (Hay, 1995) and proliferation of dwarfing genes in cereals (Hedden, 2003).

The rationale for improving photosynthesis in crop plants is grounded in the relation between photosynthesis and the efficiency of converting radiation to plant biomass (Monteith, 1977; Zhu et al., 2010). Further enhancement of carbon assimilation will increase conversion efficiency and yield potentials (Long et al., 2006; Slattery and Ort,
A number of strategies might enhance photosynthesis in C₃ species and are discussed at length elsewhere (von Caemmerer and Evans, 2010; Zhu et al., 2010; Evans, 2013; Ort et al., 2015; Niinemets et al., 2017). Briefly, these strategies fall into three general categories: (1) those that enhance the energy output of the light reactions (e.g., Kromdijk et al., 2016), (2) those that enhance the light-independent reactions, i.e., they increase carboxylation and/or ribulose-1,5-bisphosphate (RuBP) regeneration rates (e.g., Lefebvre et al., 2005; Tomeo and Rosenthal, 2017), and (3) those that would reduce photorespiration (e.g., Raines, 2006; Kebeish et al., 2007). Clearly these three strategies are not mutually exclusive and the greatest results will be achieved through coordinated improvements in all three. Indeed, the biochemical model of photosynthesis (Farquhar et al., 1980), and empirical fits to the model demonstrate that photosynthesis is commonly limited by both light and carbon supply under both natural (Wullschleger, 1993) and agronomic scenarios (Bernacchi et al., 2005; Chen et al., 2005). This co-limitation by carboxylation and RuBP-regeneration, referred to as the ‘teeter-totter’ model (Farquhar et al., 2001), is effectively maintained by partitioning nitrogen between carboxylation capacity (\(V_{C_{\text{max}}}\)) and electron transport capacity (\(J_{\text{max}}\)). A consistent ratio is preserved between \(V_{C_{\text{max}}}\) and \(J_{\text{max}}\) across differing environments (Walker et al., 2014) even though redistribution of nitrogen between the traits should enhance photosynthesis (Evans, 1989; Zhu et al., 2007; Kromdijk et al., 2016b). If \(V_{C_{\text{max}}}\) and \(J_{\text{max}}\) are genetically correlated (Geber and Dawson, 1997) it would allow for coordinated enhancement of both traits, yet it could also constrain independent selection on these traits to maximize photosynthesis.

Due to the antagonistic effects of photorespiration on net carbon assimilation, it is an obvious target for enhancing photosynthetic rates. Photorespiration occurs when two
glycolate molecules are formed from Rubisco oxygenation activity. Glycolate is toxic to plants and is metabolized via the C₂-photorespiratory pathway resulting in the return of one phosphoglycerate to the Calvin cycle, but also leads to the release of one CO₂ molecule, and consumption of both ATP and reducing equivalents (Maurino and Peterhansel, 2010; Peterhansel et al., 2010). The ratio of oxygenation to carboxylation reactions by Rubisco increases with temperature (Jordan and Ogren, 1984; Bernacchi et al., 2001), and decreases with [CO₂] providing a key benefit of the C₄ photosynthetic pathway where photorespiratory energetic losses are minimal. Alternative pathways for metabolizing glycolate have been engineered into Arabidopsis thaliana, where they successfully reduced glycolate flux through the endogenous photorespiratory pathway, enhanced net photosynthesis and increased biomass accumulation (Kebeish et al., 2007; Peterhansel and Maurino, 2011). These results have not yet been replicated in crop species or field experiments though. Extensive regulatory linkages and feedbacks exist between the photosynthetic and photorespiratory pathways (Timm et al., 2016), potentially complicating the advancement of alternative pathways to glycolate metabolism and efforts to reduce photorespiration more generally. Despite a near complete description of the photorespiratory pathway, availability and characterization of knockouts mutants for many of the enzymes in the pathway (Maurino and Peterhansel, 2010), and thorough understanding of the response of photorespiration to environmental stimuli, little is known about intraspecific genetic variation in photorespiration (Nunes-Nesi et al., 2016). Minimal within-species variation is expected to exist for Rubisco’s specificity to CO₂ relative to O₂ (Galmés et al., 2014; Prins et al., 2016), though other components affecting photorespiration may reveal meaningful variation that can be exploited. For example, genetic variation does exist for stomatal and mesophyll
conductance, which together determine the [CO₂] in the chloroplast (Soolanayakanahally et al., 2009; Tomeo and Rosenthal, 2017). Stomatal conductance also modulates leaf temperature, providing a second potential mechanism for altering the oxygenation rate of Rubisco. The degree of genetic variation for photorespiration and its relation to photosynthesis remain open questions.

Achieving drastic enhancement of photosynthesis may arise through wholesale re-engineering of the photosynthetic and photorespiratory machinery (Long et al., 2015; Ort et al., 2015). Progress is being made on some strategies (Kromdijk et al., 2016a), in many cases though, clarifying the technical details and developing the technological infrastructure leading to advanced engineering is still 10-20 years away (Zhu et al., 2010). In the interim we must continue to improve yields. A greater understanding of how natural selection altered photosynthesis and photorespiration during population expansions into new habitats and niches provides us with a framework for understanding the extent of variation and covariation among photosynthetic traits, and how we might alter them to improve photosynthetic efficiency (Flood et al., 2011). Natural populations illustrate both the extent of phenotypic space occupied by successful trait combinations and the areas of unoccupied phenotypic space resulting from unsuccessful trait combinations (Donovan et al 2011). Ample genetic variation exists for a wide array of photosynthetic traits in wild (Geber and Dawson, 1997; Brouillette et al., 2014; Momayyezi and Guy, 2016) and crop plants (Barbour et al., 2010; Giuliani et al., 2013; Gu et al., 2014a; Tomeo and Rosenthal, 2017). Even in plant biology’s premiere model species, *Arabidopsis thaliana*, photosynthetic rates and associated traits differ substantially among wild populations (Brosché et al., 2010; Easlon et al., 2014; van Rooijen et al., 2015).
The *Arabidopsis thaliana* model system holds great potential for discovering natural variation in photosynthesis and the mechanisms giving rise to that variation. Vast biochemical, genetic, and molecular knowledge and tools are available for *A. thaliana* (http://www.arabidopsis.org/), providing the requisite resources needed to dissect diverging phenotypes. An arguably underutilized resource at our disposal is the large collection of naturally occurring *A. thaliana* inbred lines, i.e. ecotypes. Ecotypes originating from across Eurasia and North America, and thus adapted to a great diversity of climates (Koornneef et al., 2004; Alonso-Blanco et al., 2016), are readily available from stock centers. The sequenced genomes of 1,135 ecotypes were recently published providing an additional tool for linking phenotypes and genotypes in this system (Alonso-Blanco et al., 2016). A number of studies have utilized *A. thaliana* ecotypes to demonstrate genetic variation in different aspects of photosynthetic physiology. Ecotypes exhibit variability in their stomatal responsiveness to [CO$_2$] (Takahashi et al., 2015) and closing stimuli (Aliniaeifard and Van Meeteren, 2014). There is standing heritable genetic variation across ecotypes for light use efficiency (i.e. quantum yield of photosystem-II) (van Rooijen et al., 2015), integrated water-use-efficiency (i.e. carbon isotope composition of leaves) (McKay et al., 2003; Easlon et al., 2014), and both stomatal conductance and transpiration efficiency (Easlon et al., 2014). A more thorough understanding of the genetic variation and correlation of photosynthetic traits will allow future studies to directly target specific traits, or suites of genetically correlated traits, for thorough characterization of the genetic architecture giving rise to that variation. For example, originally using ecotypes, a large quantity of variation for integrated water use efficiency was detected among ecotypes (McKay et al., 2003). Taking advantage of a recombinant inbred line population, five quantitative trait loci of major effect on integrated
water use efficiency were then discovered (Juenger et al., 2005). Then using inbred lines from a cross between two ecotypes with divergent water use efficiency, McKay et al., (2008) showed not only are there quantitative traits of major effect, but water use efficiency is responsive to cytoplasmic background (i.e., chloroplast genes) of lines. The availability of A. thaliana ecotypes with contrasting phenotypes, and inbred line populations derived from them, provide a powerful system for exploring natural genetic variation in photosynthetic physiology.

**Hypotheses and experimental overview**

In this study we set out to demonstrate the broad extent of natural genetic variation for leaf-level photosynthetic physiology in A. thaliana ecotypes originating from diverse geographic and climatic regions (Table 2.1). Specific ecotypes were selected to represent a range of variation in rosette-level gas-exchange parameters and carbon water use efficiency (Easlon et al., 2014). The main focus in this study is to identify the presence and magnitude (or absence) of genetic correlations between traits underlying photosynthesis and photorespiration. A growing body of evidence suggests that photosynthesis and photorespiration are co-regulated (Bauwe et al., 2012; Timm et al., 2016) leading to the hypothesis that they should be positively correlated. However, the magnitude of intraspecific variation for photorespiration is unknown. Here we hypothesize that: (i) carbon (stomatal and mesophyll conductance, or their sum) and energy supplies (as electron transport rate) to the Calvin cycle are coordinated at genetic as well as phenotypic levels, (ii) genetic variation in photorespiration exists and is positively correlated with net assimilation rates. For hypothesis (i) we specifically test for genetic correlations between stomatal conductance, mesophyll conductance, or their sum and electron transport rates, with additional validation by testing for genetic
correlation between $V_{\text{Cmax}}$ and $J_{\text{max}}$. Easlon et al. (2014) suggested that stomatal conductance was of greater consequence to water use efficiency than the carbon assimilation rate in $A. \text{thaliana}$ ecotypes, but mesophyll conductance was only implicated as driving some of the variation in water use efficiency. Our dataset utilizing leaf-level gas-exchange allow us to explicitly test the hypothesis (iii) that water use efficiency is correlated negatively with stomatal conductance, but positively with mesophyll conductance and net assimilation.

**Methods**

*Plant material*

Seeds of 14 *Arabidopsis thaliana* ecotypes (Table 2.1) were obtained from the Arabidopsis Biological Resource Center (abrc.osu.edu) and grown for one generation to collect fresh seed for all ecotypes in a common environment. The ecotypes were chosen to reflect as much variance in photosynthetic traits as possible based on previous rosette-level measurements (Easlon et al., 2014). Seeds were sown in 160 mL pots in a moist 4:1 mixture of Metro-Mix 360 topsoil (sungro Horticulture, Agawam, MA, USA) and fritted clay (Turface, Profile Products LLC, Buffalo Grove, IL, USA). Pots were placed in water inside covered trays and incubated in the dark at 4º C for seven days. Trays were then transferred to controlled environment growth chambers set to a photosynthetic photon flux density (PPFD) of approx. 400 $\mu$mol photons m$^{-2}$ s$^{-1}$, day length of 12 hours, and day:night temperature of 21:19º C. Upon germination covers were removed from trays. At the six-leaf stage pots were thinned to a single plant, and as a precautionary measure to prevent any chance of cross-pollination or seed contamination between pots, 40 cm floral sleeves were placed around the pots. Pots were rotated within trays weekly and trays were rotated within the chamber every three to four days. Water was replaced
in trays as needed and supplemented with 0.5X Hoagland's solution weekly. At 21 days post-germination winter-annual variety ecotypes were transferred to a second growth chamber set to identical conditions, excepting the temperature set to 4°C day and night, where they were kept for 28 days before returning to the original chamber. Once the majority of siliques on a plant reached maturity the plant was placed in a separate tray without water, allowed to dry for several days, and seeds were harvested.

To facilitate leaf-level gas exchange measurements on the rosette leaves of *A. thaliana*, experimental plants were grown using the 'ice-cream-cone-like' technique of Flexas et al. (2007b)(Figure 2.S1). Essentially, 160 mL pots were overfilled, with the same Metro-Mix 360 and fritted clay soil mixture used above, such that the soil formed a dome sticking out of the top of the pot. To hold the soil in place the soil was covered with fiberglass screen affixed to the pot. Seeds were then sown approx. 1 cm from the edge of the pot around the perimeter of the dome so that once mature the leaves facing outward would hang over the edge of the pot with enough length to facilitate insertion into the measurement chamber of a standard gas exchange cuvette. Plants were grown in six replicate blocks with each block contained on a single tray. Two trays (i.e. blocks) were grown simultaneously in a growth chamber set to a PPFD of approx. 450 μmol photons m−2 s−1 during the 12 hour days, day:night temperature of 21:18.5°C, and relative humidity maintained between 60-80%. The additional four blocks were grown iteratively. Initially four seeds were sown in each pot and at six to eight leaf stage were thinned to two-plants per pot arranged at opposite sides of the pot. Once leaves reached a size large enough, but always before bolting, one plant from each pot was measured.
**Gas exchange and associated measurements**

CO₂-response curves were measured at ambient and 1% O₂ (Figure 2.S2). The youngest fully expanded leaf was placed carefully into the leaf chamber of a 6400-40 fluorescence chamber (LI-6400 Photosynthesis System, LI-COR Environmental, Lincoln, NE, USA) making sure not to damage the leaf, while also to assuring contact between the leaf and thermocouple and covering as much of the 2 cm² chamber as possible. Leaves were acclimated for ≥25 minutes to a PPFD of 850 µmol photons m⁻² s⁻¹ with 10% blue light, a vapor pressure deficit below 1.2 kPa, ambient [CO₂] of 400 µmol CO₂ mol⁻¹ air, flow rate of 300 µmol air min⁻¹, and block temperature controlled to create a 25°C leaf temperature. Preliminary light response curves indicated that all ecotypes were light saturated at 650 µmol photons m⁻² s⁻¹. Upon reaching steady state conditions a data point was logged and the [CO₂] was changed iteratively along the sequence 400, 325, 250, 175, 100, 50, 400, 400, 500, 650, 950, 1250, 1600, 2000 µmol CO₂ mol⁻¹ air. After reaching stable conditions at each CO₂ set-point gas exchange parameters and steady state fluorescence (Fₛ) were logged. Before proceeding to the next [CO₂] a multiphase flash chlorophyll fluorescence routine was executed to determine the maximum (Fₘ') fluorescence following the recommended procedures (Loriaux et al., 2013). Once the initial CO₂-response curve at ambient [O₂] was complete the leaf was allowed to re-acclimate to ambient conditions until net assimilation and stomatal conductance (gₛ) returned to their steady-state values. A humidified nitrogen tank with 1% O₂ was then connected to the air inlet on the 6400 console and a second CO₂-response curve was performed with only the sub-ambient CO₂ concentrations (400, 325, 250, 175, 100, and 50 µmol mol⁻¹). At each CO₂ set-point, Fₛ and Fₘ’ were again estimated with the multiphase flash routine.
As some leaves did not completely fill the gas exchange cuvette, the calculated parameters needed to be recalculated with the appropriate leaf area. Before these leaves were taken out of the gas exchange system the location where the leaf entered the foam gaskets of the cuvette was traced on the leaf itself with a marker. After absorptance measurement (see below), the leaf was placed onto the bench top and a spare gasket was placed on top of leaf using the markings as a guide so that the gasket and leaf matched their orientations when inside the gas exchange cuvette. The portion of the leaf that was inside the cuvette was then cut out with a scalpel. A digital image of the leaf section and a ruler were captured on a white background. These images were used to calculate the area of the leaf in the gas exchange cuvette using standard procedures with ImageJ (Schneider et al., 2012). Finally, the leaf area was updated in the spreadsheet output of the 6400 to recalculate all parameters.

Following gas exchange measurement leaf absorptance (\(\alpha\)) was assessed with a spectroradiometer and integrating spheres built into a leaf clamp (Jaz Spectroclip, Ocean Optics Inc., Dundee, FL, USA). Reflectance and transmittance were assessed at three locations on the same leaf used for gas exchange. The calculation of \(\alpha\) constrained the spectroradiometer output to ±5 nm surrounding the LED peaks of the 6400-40 light source (470 and 665 nm), and was weighted to account for the light used during gas exchange being composed of 10% blue and 90% red. Absorptance was calculated as \(1-(\text{reflectance} + \text{transmittance})\).

Leaf thickness was measured with digital calipers on the leaf of the rosette directly opposite that used for gas exchange. Then this leaf and four to five additional fully expanded leaves were sampled for determination of leaf mass per area and leaf dry matter content. First the leaves were laid flat on a white background with a ruler and a
digital image was taken. Total leaf area was determined using ImageJ. The leaves were then massed for fresh weight. After drying at 65°C for >72 hours the leaves were massed again for dry weight. Leaf dry matter content was calculated as the ratio of dry to fresh mass. Leaf mass per area was calculated as the ratio of dry mass to total leaf area. Dry leaves were then ground to a fine powder and analyzed for carbon, nitrogen, and 13C content at The University of Illinois. The carbon isotope ratio (13C to 12C) of leaf tissue is reported as δ13C in units of parts per thousand (‰) and is indicative of the ratio of intercellular to atmospheric CO2 concentration integrated over the lifetime of the leaf (Farquhar et al., 1989). When leaves are sampled from a common environment (i.e., homogenous vapor pressure deficit and air [13C]), then δ13C is interpreted as an integrated measure of leaf water use efficiency with larger δ13C indicating higher water use efficiency (Farquhar et al., 1989).

Analysis of gas exchange data

Leaks are a substantial problem in standard gas exchange systems and can result in systematic biases due to the contrasting diffusional gradients at opposing ends of a CO2-response curve (Flexas et al., 2007a; Rodeghiero et al., 2007; Gong et al., 2015). As a precaution against these biases we have measured the apparent-photosynthesis throughout identical CO2-response curves on heat-inactivated (n=13) leaves with petioles kept in water to maintain hydration. Apparent-photosynthesis of these leaves was then subtracted from all measured CO2-response curves, followed by updating the estimates of intercellular-[CO2] (Ci) given the new net-assimilation rates (Flexas et al., 2007a; 2012). While heat-inactivated leaves do not perfectly match the characteristics of living leaves, they should mimic the characteristics of leaves better than wet filter paper (Flexas et al., 2007a), and many of the diffusional leaks in the 6400 system likely
upstream of the leaf chamber and therefore will be equally accounted for despite the material used to mimic intact leaves.

CO$_2$-response curves were initially fit to the C$_3$ biochemical model of photosynthesis (Farquhar et al., 1980) to estimate mitochondrial respiration in the light ($R_d$). Each curve was fit using the R package plantecophys (Duursma, 2015) and the estimate for $R_d$ was extracted. Mean-ecotype $R_d$ values were calculated from individual curves and used in further calculations below.

Electron transport rates through photosystem II (PSII) measured with combined gas-exchange and chlorophyll fluorescence support alternative electron sinks, photosynthesis, and the photorespiratory pathway. If corrections for alternative electron sinks are implemented, we can partition the remaining electrons to their respective destinations in photosynthesis and photorespiration. We first quantified the quantum yields of CO$_2$-fixation ($\Phi_{CO2}$) and PSII ($\Phi_{PSII}$) (Valentini et al., 1995; Long and Bernacchi, 2003) as:

$$\Phi_{CO2} = \frac{A_N + R_d}{\alpha PPFD}$$ \hspace{1cm} \text{Equation 2.1}

$$\Phi_{PSII} = \frac{F_m' - F_s}{F_m'}$$ \hspace{1cm} \text{Equation 2.2}

where $A_N$ is the leak corrected net assimilation rate, $\alpha$ is the leaf absorptance measured as above; PPFD, $F_m'$, and $F_s$ were taken from the 6400 output. The relationship of $\Phi_{CO2}$ and $\Phi_{PSII}$ under non-photorespiratory conditions (1% O$_2$) should be linear (Genty et al., 1989) with the intercept representing the share of electrons destined to alternative sinks and the slope indicating the number of electrons required for reducing a CO$_2$ molecule in vivo. Assuming that this relationship holds under photorespiratory conditions $\Phi_{PSII}$ was calibrated ($\Phi_e$; Valentini et al., 1995) as:

At 1% O$_2$: \hspace{1cm} $\Phi_{PSII} = k\Phi_{CO2} + b$ \hspace{1cm} \text{Equation 2.3}
At 21% O$_2$: $\Phi_e = \frac{4(\Phi_{PSII} - b)}{k}$  

Equation 2.4

where $k$ and $b$ are respectively the slope and intercept of the linear regression, and four is the theoretical number of electrons required for a single carboxylation (Long and Bernacchi, 2003). $\Phi_e$ in Equation 4 represents the quantum efficiency of PSII corrected for any alternative electron sinks. With $\Phi_e$ we calculated the total electron flux through PSII used to support both photosynthesis and photorespiration ($J_T$):

$$J_T = \Phi_ePPFD$$  

Equation 2.5

and

$$J_T = J_C + J_O$$  

Equation 2.6

where $J_C$ and $J_O$ are the electron flows to carboxylation and oxygenation respectively.

Then assuming that four electrons are required for each CO$_2$ carboxylation, and eight electrons for each CO$_2$ release following an oxygenation event:

$$J_C = 4(A_N + R_d + PR)$$  

Equation 2.7

$$J_O = 8PR$$  

Equation 2.8

where $PR$ is the photorespiratory CO$_2$ release rate, calculated as:

$$PR = \frac{[J_T - 4(A_N + R_d)]}{12}$$  

Equation 2.9

We can insert Equation 9 into Equations 7 and 8 to partition electrons between the two pathways:

$$J_C = \frac{[J_T + 8(A_N + R_d)]}{3}$$  

Equation 2.10

$$J_O = \frac{2[J_T - 4(A_N + R_d)]}{3}$$  

Equation 2.11

A reliable estimate of $J_T$ also allows for the calculation of mesophyll conductance ($g_m$) with the variable-J method (Harley 92):

$$g_m = \frac{A_N}{\{C_i - [\Gamma^*(J_T + 8(A_N + R_d)) / [J_T - 4(A_N + R_d)]\}}$$  

Equation 2.12

where $\Gamma^*$ is the photosynthetic CO$_2$ compensation point in the absence of mitochondrial respiration in the light ($R_d$). There are many values of $\Gamma^*$ in the literature for A. thaliana,
ranging from 3.3 to 5.4 Pa (Kebeish et al., 2007; Flexas et al., 2007b; Cousins et al., 2011; Walker et al., 2013; Walker and Cousins, 2013; Weise et al., 2015). Mesophyll conductance is sensitive to variation in $\Gamma^*$ (Pons et al., 2009) so we estimated $g_m$ with $\Gamma^*$ values of 3.64 (Walker et al., 2013) and 4.47 (Weise et al., 2015) since these fall within, but span most of the range in estimates. Estimates of $g_m$ were greater when estimated with $\Gamma^*$ of 4.47 Pa. The correlation of $g_m$ estimates with $\Gamma^*$ of 3.64 or 4.47 Pa was high ($r=***$) and no differences in the resulting genetic variance of $g_m$ were detected. Therefore, we chose to use the value of $\Gamma^*$ from Walker et al. (2013) since it falls in between the extremes of the other two estimates. Theoretically, with the variable-$J$ method $g_m$ can be estimated at any intercellular CO$_2$ concentration ($C_i$) where $A_n$ is carboxylation limited. However, since $g_m$ is often observed to vary with $C_i$ we estimated $g_m$ from observations at a common, near-ambient [CO$_2$] of 325 µmol mol$^{-1}$ as carboxylation should be limiting at this concentration; note though that we saw no significant difference in estimates at ambient [CO$_2$]'s of 325 or 400 µmol mol$^{-1}$. With a known $g_m$ the [CO$_2$] in the chloroplast ($C_c$) was calculated as:

$$C_c = C_i - A_n / g_m$$

Equation 2.13

Finally, with values of $C_c$ we again fit the biochemical model of C$_3$ photosynthesis (Farquhar et al., 1980) using the plantecophys package (Duursma, 2015) to estimate the maximum carboxylation capacity ($V_{C_{\text{max}}}$) and maximum electron transport capacity ($J_{\text{max}}$). Since we are unable to assure that PPFD was saturating for all $A_n$-$C_i$ curves measured, $J_{\text{max}}$ is reported as $J_{850}$, i.e. subscripted with the measurement PPFD of 850 µmol photons m$^{-2}$ s$^{-1}$ (Buckley and Diaz-Espejo, 2015).
Statistical analysis

All computations and statistical analyses were performed with the R statistical computing environment (R Core Team, 2015). Models with ecotype and replication block as random effects were fit with the \texttt{lmer} function (Bates et al., 2015) for each trait using restricted maximum likelihood. Significance ($p<0.05$) of ecotype was determined by likelihood ratio tests between models with and without the ecotype effect. Given the number of traits investigated, we corrected all $p$-values to account for multiple testing using the false discovery rate (Benjamini and Hochberg, 1995).

For traits with a significant ecotype effect, genetic variation for each trait was then determined by partitioning variance to ecotype ($V_G$), replication block ($V_E$), and residual variation ($V_R$). Broad-sense heritability ($H^2$) was calculated as $H^2 = V_G / (V_G + V_E/6 + V_R)$, where $V_E$ was divided by six to account for the six replicate blocks in the experimental design. Additionally, for each trait with a significant ecotype effect we calculated best linear unbiased predictors (BLUPs) as the model-predicted ecotype values. Phenotypic correlations between traits were calculated as Pearson product-moment correlations of all observations of the two traits. Likewise, genetic correlations were calculated with Pearson product-moment correlations of trait BLUPs. Again, $p$-values were adjusted to account for the large number of comparisons (Benjamini and Hochberg, 1995). To compare trait-values in ecotypes with spring vs. winter growth habits we performed Welch’s unequal variance $t$-tests to account for the unequal sample sizes.

Results

We observed substantial phenotypic and genotypic variation for leaf-level physiological traits in the fourteen Arabidopsis thaliana ecotypes assessed here. Phenotypically stomatal conductance to CO$_2$ ($g_{sc}$; see Table 2.2 for all abbreviations and
their units) and mesophyll conductance to CO$_2$ ($g_m$) differed 2.4- and 2.5-fold respectively across all observations (ranging 0.0724-0.259 µmol CO$_2$ m$^{-2}$ s$^{-1}$, and 0.356-1.20 µmol CO$_2$ m$^{-2}$ s$^{-1}$ Pa$^{-1}$), while genotype (i.e., variation across ecotype and estimated with best-linear-unbiased-predictors (BLUPs)) accounted for 57 and 41% of the total variance for $g_{sc}$ and $g_m$, respectively. Conductance through the full CO$_2$-diffusion path ($g_{tot}$) varied 1.8-fold phenotypically (range: 0.0248-0.0707 µmol CO$_2$ m$^{-2}$ s$^{-1}$) and 0.4-fold genotypically. Reductant supply to the photosynthetic reactions similarly varied though at lower magnitudes. Total, calibrated, linear-electron transport ($J_T$) rates varied 1.4-fold phenotypically (range: 62.7-150 µmol e$^-$ m$^{-2}$ s$^{-1}$), and 21% genotypically. Total electron transport was partitioned to electrons destined for use in carboxylation ($J_C$) and oxygenation ($J_O$), which varied 1.4- and 0.80-fold phenotypically (ranging 42.2-99.8 and 19.0-57.5 µmol e$^-$ m$^{-2}$ s$^{-1}$, respectively), 19% and 29% genotypically, respectively. It follows that net assimilation rates ($A_n$) differed 1.6-fold phenotypically (range: 6.97-18.3 µmol CO$_2$ m$^{-2}$ s$^{-1}$), and 21% genotypically. Photorespiration ($PR$) also varied, just over 2-fold phenotypically (range: 2.38-7.19 µmol CO$_2$ m$^{-2}$ s$^{-1}$) and 29% genotypically. As implied by the presence of genotypic variation, broad-sense heritabilities were significant for all traits above (Table 2.3).

Leaf structural traits and water use efficiency traits also varied among ecotypes. Roughly half of the variation in both leaf mass per area ($LMA$) and leaf dry matter content ($LDMC$) were controlled by genetics with 46 and 39% of variation among ecotypes for the two traits that varied approx. 1- and 0.75-fold phenotypically. Intrinsic water use efficiency ($A_n/g_s$) differed 1.5-fold across all observations and 42% (i.e., 21 µmol CO$_2$ mol H$_2$O) among ecotypes. Leaf carbon isotope composition ($\delta^{13}C$) differed 4.8 ‰ among observations and 2.9 ‰ among BLUPs. Broad-sense heritabilities were
generally higher for water use efficiency and structural traits than for photosynthetic traits (Table 2.3).

Physiological leaf traits were by and large coordinated with one another both phenotypically and genotypically (Table 2.4). Phenotypically the traits supplying the Calvin cycle with CO₂, i.e., stomatal conductance \((g_{sc})\) and mesophyll conductance \((g_m)\), were correlated \((r_p=0.49, p<0.001)\), though the genetic correlation was not significant \((p>0.1)\). As expected, all CO₂ conductance traits \((g_{sc}, g_m, \text{ and } g_{tot})\) were correlated with net photosynthetic rate \((A_N)\), except the genetic correlation with \(g_{sc}\) (Figure 2.1). Phenotypic and genetic correlations between total electron transport \((J_T)\) and \(A_N\) were both significant \((r_p=0.86, r_g=0.79, p<0.001\) for both), but were lower than the correlation with \(A_N\) after partitioning electrons specifically to carboxylation, i.e. \(J_C\) \((r_p=0.96, r_g=0.92, p<0.001\) for both). Both \(g_{sc}\) and \(g_m\) were phenotypically correlated with \(J_T\) \((g_{sc}: r_p=0.37, p<0.01; g_m: r_p=0.67, p<0.001)\) and \(J_C\) \((g_{sc}: r_p=0.49, p<0.001; g_m: r_p=0.81, p<0.001)\) (Figure 2.2 A), but only \(g_m\) was genetically correlated with electron transport \((J_T: r_g=0.66, p<0.05; J_C: r_g=0.81, p<0.01)\). Maximum biochemical capacity for carboxylation by Rubisco \((V_{cmax})\) and maximum capacity for electron transport \((J_{850})\) were tightly correlated both phenotypically \((r_p=0.90, p<0.001)\) and genetically \((r_g=0.92, p<0.001)\) (Figure 2.2 B).

To estimate photorespiration \((PR)\) we partitioned the portion of \(J_T\) used to support \(PR\) \((J_O)\), and then calculated \(PR\) from \(J_O\). While \(J_O\) and \(PR\) represent different aspects of physiology they are calculated from the same combination of traits (i.e., \(A_N, R_d\), and \(J_T\)), only differing with respect to the stoichiometric coefficients used in their calculation such that \(J_O\) and \(PR\) are directly proportional to one another. Therefore comparisons with both traits yield equivalent statistical results - albeit with differing parameter estimates – but
since we are interested specifically in photorespiration, we chose to present results for PR only. We detect correlation between $A_N$ and PR at the phenotypic level ($r_p=0.64$, $p<0.001$; Figure 2.3); the genetic correlation was not significant after controlling for multiple testing ($r_g=0.53$, $0.1>p>0.05$). Since $A_N$ is an input parameter in the calculation of PR, some correlation of $A_N$ and PR is expected. We further test of physiological correlation of photosynthesis with photorespiration by comparing the biochemical parameter underlying photosynthetic capacity, i.e., $V_{\text{Cmax}}$ and $J_{850}$, with PR. The phenotypic correlations of PR with $V_{\text{Cmax}}$ ($r_p=0.53$, $p<0.001$) and $J_{850}$ ($r_p=0.49$, $p<0.001$) were lower than with $A_N$ directly, but a clear positive relationship remained (Figure 2.3). No genetic correlations between PR and $V_{\text{Cmax}}$ or $J_{850}$ were detected (Table 2.4).

Leaf level water use efficiency is determined by $A_N$, $g_s$, and $g_m$. We assess the extent that each of these traits contributes individually to intrinsic water use efficiency ($A_N/g_s$) and integrated water use efficiency ($\delta^{13}C$). Both metrics of water use efficiency were phenotypically correlated ($r_p=0.67$, $p<0.001$) and demonstrated an even stronger genetic correlation with each other ($r_g=0.971$, $p<0.001$) (Figure 2.4A). The phenotypic ($r_p=-0.67$, $p<0.001$) and genetic ($r_g=-0.96$, $p<0.001$) correlations of $\delta^{13}C$ with the ratio of intercellular to ambient [CO$_2$] ($C_i/C_a$) mirrored those with $A_N/g_s$ (Figure 2.4B). Net assimilation rates were not correlated with $A_N/g_s$ and only a weak phenotypic correlation was found with $\delta^{13}C$ ($r_p=0.276$, $p<0.05$) (Table 2.4; Figure 2.5 A&D). Evidence of $g_s$ influencing water use efficiency was stronger: phenotypic and genetic correlations were observed between $g_s$ and $A_N/g_s$ ($r_p=-0.69$, $p<0.001$; and $r_g=-0.71$, $p<0.05$) and $\delta^{13}C$ ($r_p=-0.36$, $p<0.01$; and $r_g=-0.71$, $p<0.05$) (Table 2.4; Figure 2.5 B&E). The influence of $g_m$ mirrored that of $A_N$ in that it was only weakly correlated phenotypically with $\delta^{13}C$ ($r_p=0.276$, $p<0.05$) (Table 2.4; Figure 2.5 C&F). Full-path CO$_2$ conductance, $g_{\text{tot}}$, was not
associated with either metric of water use efficiency (Table 2.4). Ecotype growth habit (spring or winter annual type) altered relationships with water use efficiency. The correlations of $\delta^{13}$C with $A_{N}$ and $g_{m}$ were only significant for winter annual types (Figure 2.5 D&F), and with $g_{s}$ only for spring types (Figure 2.5E).

The ecotypes used in this study originate from areas spanning a large latitudinal range, and thus areas differing widely in variables that covary with latitude (e.g., temperature and precipitation; Table 2.1). Ecotypes exhibit winter and spring annual life history strategies, and with the exception of Kas-1, winter annual types tend toward higher latitudes (Table 2.1). Winter annual types had higher $g_{m}$ ($t=2.7$, $p<0.05$; not shown), $N_{\text{area}}$ ($T=6.1$, $p<0.001$), $V_{\text{Cmax}}$ ($t=3.1$, $p<0.01$), $J_{850}$ ($t=3.1$, $p<0.001$), $LMA$ ($t=7.7$, $p<0.001$), and water use efficiency ($A_{N}/g_{s}$: $t=4.3$, $p<0.001$; $\delta^{13}$C: $t=5.7$, $p<0.001$)(Figure 2.6).

**Discussion**

**Genetic variation in photosynthetic physiology**

Natural genetic variation for photosynthetic traits holds potential as a continued source of useful trait variation for agronomic improvements. The most ancient means of crop improvement is still a viable strategy when heritable variation exists in desirable traits. Likewise, natural genetic variation can be mined to expand our understanding of this most important of biochemical pathways (Alonso-blanco et al., 2009). Genetic variation for photosynthetic traits is commonly observed in wild species (Comstock and Ehleringer, 1992; Geber and Dawson, 1997; Arntz and Delph, 2001; Geber and Griffen, 2003; Brouillette et al., 2014), crops (Reynolds et al., 2000; Gu et al., 2012; Liu et al., 2012; Tomeo and Rosenthal, 2017), and specifically in *A. thaliana* (McKay et al., 2003; Aliniaieifard and Van Meeteren, 2014; Easlon et al., 2014; van Rooijen et al., 2015;
Takahashi et al., 2015). The study here adds to this literature by demonstrating heritable variation for photorespiration in addition to photosynthesis itself (Table 2.3).

**Coordination of photosynthetic drivers**

Across the *Arabidopsis thaliana* ecotypes there was broad coordination among photosynthetic traits. We expect to see phenotypic correlations among traits supporting photosynthesis due to the necessity of optimizing resource supplies driving the Calvin cycle. It is well established that $V_{C_{\text{max}}}$ and $J_{\text{max}}$ are coordinated at various scales reflecting optimization of nitrogen distribution among photosynthetic proteins to balance photosynthetic limitation between carboxylation and ribulose 1,5-bisphosphate (RuBP) regeneration (Wullschleger, 1993; Geber and Dawson, 1997; Walker et al., 2014). Thus, coordination should be expected between traits affecting CO$_2$ diffusion to Rubisco (i.e., $g_{sc}$, $g_{m}$, $g_{tot}$) and rates of electron transport through the light-dependent reactions of photosynthesis (i.e., $J_{T}$, $J_{C}$). Indeed $g_{sc}$, $g_{m}$, $J_{T}$, $J_{C}$, $J_{850}$, and $V_{C_{\text{max}}}$ were all phenotypically intercorrelated (Table 2.3). Both $g_{m}$ vs. $J_{C}$, and $V_{C_{\text{max}}}$ vs. $J_{850}$, also exhibited strong genetic correlations. This indicates that the essential coordination of the photosynthetic light-dependent and light-independent reactions supporting the co-limitation of photosynthesis by RuBP carboxylation and regeneration is not simply a product of optimal allocation (Evans, 1989; Hikosaka and Terashima, 1995), but rather is constrained by genetics, or alternatively has been constrained by selection (Donovan et al., 2011). Genetic correlations result from pleiotropy or linkage disequilibrium, and while we cannot disentangle the contribution of each of these mechanisms with our experimental design this experimental system provides a path for doing so in future studies. Regardless of the mechanism, the strength of the genetic correlation between
V_{\text{Cmax}} \text{ and } J_{850} \text{ here, and in at least one other study (Geber and Dawson, 1997), suggest that selection on either trait would simultaneously alter the other.}

**Correlation of photosynthesis and photorespiration**

At the molecular level the Calvin cycle and photorespiratory pathway are interlinked to such an extent that they are referred to holistically as a ‘supercycle’ (Timm et al., 2016). The 2-phosphoglycolate (2-PG), formed from Rubisco oxygenation of RuBP, inhibits two Calvin cycle enzymes, triosephosphate isomerase and phosphofructokinase (Bauwe et al., 2012), and interferes with the breakdown of starch (Kelly & Latzko 1976). If flux through the photorespiratory pathway is too slow, the concentration of 2-PG will rise until it inhibits regeneration of RuBP through the Calvin cycle and ultimately suppresses carbon-fixation (Igamberdiev et al., 2004; Bauwe et al., 2012). In addition to this feedback regulation through 2-PG, there is some evidence that the photorespiratory intermediates glyoxylate (Chastain and Ogren, 1989) and glycerate (Schimkat et al., 1990) act as signals to the Calvin cycle allowing rapid co-regulation of the two pathways (Timm et al., 2016). This molecular cross talk between the Calvin cycle and the photorespiratory pathway indicates that the two processes should manifest at the physiological scale as coordination between photorespiratory CO$_2$ efflux ($PR$) and photosynthetic CO$_2$-fixation. Indeed, that is what we observed here. The rate of photorespiratory CO$_2$ efflux was phenotypically correlated with photosynthetic carbon assimilation, and with maximum capacities for carboxylation and electron transport (i.e., $V_{\text{Cmax}} \text{ and } J_{850}$; Figure 2.3). There was significant broad-sense heritability for both $A_n$ and $PR$, though no shared inheritance for the two traits was detected. Since no significant genetic correlations were detected between $PR$ and $A_n$ (or $V_{\text{Cmax}}$, or $J_{850}$), selection to enhance $A_n$ would not necessarily also increase $PR$, or alternatively selection to reduce...
PR would not necessarily alter $A_N$, suggesting that uncoupling these traits through traditional breeding approaches is plausible. Though, it seems likely the phenotypic correlation of $A_N$ with PR arises from the interlinked nature of the two pathways at the molecular level, i.e., at higher rates of carboxylation, oxygenation is also higher and necessitating greater flux through the photorespiratory pathway to guard against inhibition of RuBP-regeneration by 2-PG. If that is the case, it suggests that efforts to lower the impact of PR on photosynthetic efficiency should remain focused on strategies to decrease the proportion of Rubisco oxygenation events, by concentrating CO$_2$ at Rubisco for example (Lieman-Hurwitz et al., 2003; Hibberd et al., 2008; von Caemmerer et al., 2012).

Drivers of variation in water use efficiency

Variation in leaf-level water use efficiency emerges from the complex interaction of a suite of traits; therefore if we wish to select for optimized physiologies it is critical to understand the relative contributions of each trait to water use efficiency. We expected to see $g_m$ positively correlated with intrinsic water use efficiency ($A_N/g_s$) due to the positive relationship between $A_N$ and $g_m$. Since $A_N$ itself was unrelated to $A_N/g_s$, it follows that both $g_m$ and $V_{C_{max}}$ also lacked influence on $A_N/g_s$. A strong case has been made for greater $g_m$ driving greater $A_N/g_s$ (Flexas et al., 2013), though this rationale is dependent on $A_N$ being a strong driver of variation in $A_N/g_s$. A previous study in A. thaliana (Easlon et al., 2014), and one in soybean (Tomeo & Rosental 2017) indicate that in herbaceous annual species with relatively high $g_s$, intrinsic water use efficiency is determined to a greater extent by variation in $g_s$ than $A_N$ under non-stressed conditions. Alternatively, it may be the case that since $A_N$ is itself correlated with $g_s$, any variation in $g_s$ has twofold
influence over $A_n/g_s$ by altering both the numerator and denominator of intrinsic water use efficiency.

Several of the complications in interpreting intrinsic water use efficiency result from it being a ratio of covarying traits; these complications are avoided by instead analyzing integrated ($i.e., \delta^{13}C$, the stable carbon isotope ratio of dry leaf tissue relative to a standard carbon source) water use efficiency. With leaves grown in the same environment, leaf $\delta^{13}C$ is indicative of discrimination against $^{13}C$, which relates to the ratio of intercellular to ambient $[CO_2]$ ($C_i/C_a$) over the lifetime of carbon accumulation in the leaf (Farquhar et al., 1982; Dawson et al., 2002; Seibt et al., 2008). The $C_i/C_a$ ratio is related to $A_n/g_s$ because any change in either $A_n$ or $g_s$ will alter $C_i$ independent of $C_a$, leading to the interpretation of $\delta^{13}C$ as proportional to the mean leaf-lifetime water use efficiency. Variation for $\delta^{13}C$ in A. thaliana is well documented in the literature among ecotypes (Nienhuis et al., 1994; McKay et al., 2003; Easlon et al., 2014) and inbred lines (Nienhuis et al., 1994; Juenger et al., 2005). As in other studies (Schuster et al., 1992; Easlon et al., 2014), broad-sense heritability for $\delta^{13}C$ was high among the ecotypes (Table 2.2); and $\delta^{13}C$ was correlated closely with both $A_n/g_s$ and $C_i/C_a$ (Figure 2.4).

Considering all ecotypes, regardless of growth habit, our results are consistent with others (Easlon et al., 2014), $\delta^{13}C$ is more closely correlated with $g_s$ than with $A_n$. Growth habit though alters the interpretation of which traits drive $\delta^{13}C$. Among ecotypes with the spring annual habit, only $g_s$ was significantly related to $\delta^{13}C$. For those with winter annual habits, $g_s$ was not correlated with $\delta^{13}C$, but $g_m$ and $A_n$ were both positively correlated with $\delta^{13}C$ (Figure 2.5). Water use efficiency is well described as a tradeoff with respect to the time required to transition from vegetative to reproductive growth in A. thaliana (McKay et al., 2003) and another annual weedy plant Polygonum arenastrum.
(Geber, 1990; Geber and Dawson, 1990). In both species there is a continuum of traits conferring a drought avoidance to drought tolerance tradeoff: fast developing genotypes have lower water use efficiency, reach the reproductive transition quickly and avoid drought, whereas slower growing genotypes have greater water use efficiency allowing them to more effectively tolerate drought (Geber, 1990; Geber and Dawson, 1990; McKay et al., 2003). Ecotypes with the winter habit in this study exhibited a greater phenotypic and genotypic range of water use efficiency (as $A_n/g_s$ or $\delta^{13}$C; Figure 2.5 & Figure 2.6), therefore sampling the phenotypic space of the drought avoidance vs. tolerance axis more effectively. The added variance in the water use efficiency traits was modulated by roughly equal ranges of $A_n$ and $g_m$ for winter and spring ecotypes, but winter ecotypes presented approximately half the range in $g_s$. Several studies have suggested that selection to improve water use efficiency by enhancing $g_m$ is contingent upon maintaining $g_s$ relatively constant (Flexas et al., 2013; Flexas et al., 2016), with a report on soybean (Glycine max) cultivars indicating that a strong correlation between $g_s$ and $g_m$ likely precludes altering water use efficiency through selection on $g_m$ (Tomeo and Rosenthal, 2017). This proposed route to selecting for a range of water use efficiencies though, appears to have already occurred among A. thaliana ecotypes.

Growth habit impacted trait values and altered relationships. On average winter habit ecotypes had greater structural robustness ($T_L$, $LMA$, and $LDMC$), water use efficiency as $A_n/g_s$ or $\delta^{13}$C, and photosynthetic capacity ($N_{area}$, $g_m$, $V_{cmax}$, and $J_{850}$). We hypothesize that thicker leaves in A. thaliana arise due to a thicker palisade mesophyll layer, which allows for a larger area of mesophyll cell surface exposed to intercellular airspace (Milla-Moreno et al., 2016), and therefore more parallel diffusion paths for CO$_2$ into mesophyll cells, explaining the greater $g_m$. The thicker leaves of winter habit ecotypes led to greater
LMA, and higher LMA is consistent with a greater number of mesophyll cells per unit leaf area allowing for higher \(N_{\text{area}}\), \(V_{\text{Cmax}}\), and \(J_{850}\). The relatively small number of ecotypes analyzed here and the imprecise geographic locations from which they were originally collected (Table 2.1) limits statistical power to interpret the correlations of trait values with climate or latitude of origin. However, trait patterns in our study are similar to those observed in *Populus balsamifera* with greater assimilation rates, electron transport, and mesophyll conductance in genotypes from more northern latitudes, and a positive correlation between latitude and water use efficiency (Soolanayakanahally et al., 2009). This increase in LMA with latitude is consistent with photosynthetic acclimation to temperature. When grown at low temperatures LMA tends to increase, effectively enhancing photosynthetic capacity per unit leaf area (Atkin et al., 2006), allowing for positive carbon balance despite the absolute decline in photosynthesis with decreasing temperatures.

**Conclusions**

*Arabidopsis thaliana* ecotypes provide a powerful system for dissecting the mechanistic and genetic determinants of complex traits. *In vivo* estimates of photorespiration indicate that the molecular coordination of the Calvin cycle with the photorespiratory pathway scales to the physiological level as a correlation between photorespiratory CO\(_2\) efflux and photosynthetic carbon assimilation. Strong genetic correlations between mesophyll conductance and electron transport supporting carboxylation, and between maximum carboxylation and electron transport capacities, point to shared inheritance for traits underlying variation in photosynthesis among ecotypes. However, if the absence of genetic correlation between photosynthesis and photorespiration in *A. thaliana* is conserved in other taxa, then independent selection on
these traits through traditional breeding strategies can both reduce photorespiration and enhance photosynthesis.

References


mesophyll conductance between barley genotypes, and effects on transpiration
Bauwe H, Hagemann M, Kern R, Timm S (2012) Photorespiration has a dual origin and
Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and
air [CO2] enrichment (FACE) stimulates photosynthesis while decreasing in vivo
Rubisco capacity. Planta 220: 434–446
temperature response functions for models of Rubisco-limited photosynthesis. Plant
Cell Environ 24: 253–259
related to resource use in a desert annual along a resource gradient. New Phytol
201: 1316–1327
transport rate. New Phytol 205: 14–7
589–592


Oecologia 78: 9–19
Farquhar GD, von Caemmerer S, Berry JA (1980) A Biochemical Model of
Photosynthetic CO2 Assimilation in Leaves of C3 Species. Planta 149: 78–90
Physiol 125: 42–5
discrimination and the intercellular carbon dioxide concentration in leaves. Funct
Plant Biol 9: 121–137
Flexas J, Díaz-Espejo A, Berry JA, Cifre J, Galmés J, Kaldenhoff R, Medrano H, Ribas-
Carbó M (2007a) Analysis of leakage in IRGA’s leaf chambers of open gas
exchange systems: quantification and its effects in photosynthesis
Flexas J, Díaz-Espejo A, Conesa MA, Coopman RE, Douthe C, Gago J, Gallé A,
and Rubisco as targets for improving intrinsic water use efficiency in C3 plants.
Flexas J, Niinemets Ü, Gallé A, Barbour MM, Centritto M, Diaz-Espejo A, Douthe C,
CO2 as a target for increasing photosynthesis and photosynthetic water-use
efficiency. Photosynth Res 117: 45–59


Controlled by a Single Dominant Gene. Crop Sci 46: 1606


Schimkat D, Heineke D, Heldt HW (1990) Regulation of sedoheptulose-1,7-bisphosphatase by sedoheptulose-7-phosphate and glycerate, and of fructose-1,6-bisphosphatase by glycerate in spinach chloroplasts. Planta 181: 97–103


accessions of Arabidopsis thaliana. New Phytol 177: 419–427


Table 2.1

All of the ecotypes used in this study are listed here. The names, accession numbers, and latitude/longitude of origin are referenced to the Arabidopsis Biological Resource Center. Mean annual temperature (MAT) and mean annual precipitation (MAP) were taken from the bioclim database (www.worldclim.org) for the given latitude/longitude locations with help from the rgdal and raster R packages. Habit refers to the life history strategy of ecotypes: winter habits require vernalization for flowering.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession</th>
<th>Latitude</th>
<th>Longitude</th>
<th>MAT</th>
<th>MAP</th>
<th>Habit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag-0</td>
<td>CS76430</td>
<td>45.0000</td>
<td>1.3000</td>
<td>11.8</td>
<td>887</td>
<td>Winter</td>
</tr>
<tr>
<td>Bil-5</td>
<td>CS76709</td>
<td>63.3240</td>
<td>18.4840</td>
<td>2.9</td>
<td>615</td>
<td>Winter</td>
</tr>
<tr>
<td>Bur-0</td>
<td>CS76734</td>
<td>54.1000</td>
<td>-6.2000</td>
<td>9.3</td>
<td>953</td>
<td>Winter</td>
</tr>
<tr>
<td>Eden-1</td>
<td>CS76826</td>
<td>62.8770</td>
<td>18.1770</td>
<td>3.3</td>
<td>655</td>
<td>Winter</td>
</tr>
<tr>
<td>Kas-1</td>
<td>CS903</td>
<td>35.0000</td>
<td>77.0000</td>
<td>-9.1</td>
<td>74</td>
<td>Winter</td>
</tr>
<tr>
<td>Knox-18</td>
<td>CS76530</td>
<td>41.2816</td>
<td>-86.6210</td>
<td>9.7</td>
<td>975</td>
<td>Spring</td>
</tr>
<tr>
<td>Ler-1</td>
<td>CS77021</td>
<td>47.9840</td>
<td>10.8719</td>
<td>8.0</td>
<td>962</td>
<td>Spring</td>
</tr>
<tr>
<td>NFA-10</td>
<td>CS77126</td>
<td>51.4108</td>
<td>-0.6383</td>
<td>9.8</td>
<td>701</td>
<td>Spring</td>
</tr>
<tr>
<td>Omo2-3</td>
<td>CS77149</td>
<td>56.1400</td>
<td>15.7800</td>
<td>7.9</td>
<td>546</td>
<td>Winter</td>
</tr>
<tr>
<td>Sq-8</td>
<td>CS76604</td>
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<td>-0.6383</td>
<td>9.8</td>
<td>701</td>
<td>Spring</td>
</tr>
<tr>
<td>Tamm-2</td>
<td>CS76610</td>
<td>60.0000</td>
<td>23.5000</td>
<td>5.1</td>
<td>611</td>
<td>Winter</td>
</tr>
<tr>
<td>Ts-1</td>
<td>CS76615</td>
<td>41.7194</td>
<td>2.9306</td>
<td>16.1</td>
<td>644</td>
<td>Spring</td>
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<tr>
<td>Tsu-1</td>
<td>CS77390</td>
<td>34.4300</td>
<td>136.3100</td>
<td>14.9</td>
<td>2385</td>
<td>Spring</td>
</tr>
<tr>
<td>Ws-2</td>
<td>CS76631</td>
<td>52.3000</td>
<td>30.0000</td>
<td>6.7</td>
<td>624</td>
<td>Spring</td>
</tr>
</tbody>
</table>

1Available Latitude and Longitude are coarse for this ecotype. Due to the rugged terrain surrounding this location and the high variability in the local climate over small spatial scales, these climate values represent rough approximations.
Table 2.2

List of all trait abbreviations used and their units.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Abbreviation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient CO(_2) concentration</td>
<td>(C_a)</td>
<td>(\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air})</td>
</tr>
<tr>
<td>Intercellular CO(_2) concentration</td>
<td>(C_i)</td>
<td>(\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air})</td>
</tr>
<tr>
<td>Chloroplast CO(_2) concentration</td>
<td>(C_c)</td>
<td>(\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air})</td>
</tr>
<tr>
<td>Stomatal conductance to water vapor</td>
<td>(g_s)</td>
<td>(\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Stomatal conductance to CO(_2)</td>
<td>(g_{sc})</td>
<td>(\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Mesophyll conductance</td>
<td>(g_n)</td>
<td>(\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1})</td>
</tr>
<tr>
<td>Total CO(_2) conductance</td>
<td>(g_{tot})</td>
<td>(\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Total linear electron transport</td>
<td>(J_T)</td>
<td>(\mu\text{mol e}^{-1} \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Electron transport to carboxylation</td>
<td>(J_C)</td>
<td>(\mu\text{mol e}^{-1} \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Electron transport to oxygenation</td>
<td>(J_O)</td>
<td>(\mu\text{mol e}^{-1} \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Maximum carboxylation capacity</td>
<td>(V_{C_{\text{max}}})</td>
<td>(\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Maximum electron transport capacity</td>
<td>(J_{\text{max}}) ((J_{850}))</td>
<td>(\mu\text{mol e}^{-1} \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Net assimilation rate</td>
<td>(A_N)</td>
<td>(\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Photorespiratory CO(_2) efflux rate</td>
<td>(PR)</td>
<td>(\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})</td>
</tr>
<tr>
<td>Intrinsic water use efficiency</td>
<td>(A_{\text{N}]/g_s)</td>
<td>(\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O})</td>
</tr>
<tr>
<td>Integrated water use efficiency</td>
<td>(\delta^{13}\text{C})</td>
<td>(%)</td>
</tr>
<tr>
<td>Leaf nitrogen content</td>
<td>(N_{\text{mass}})</td>
<td>%</td>
</tr>
<tr>
<td>Nitrogen per unit leaf area</td>
<td>(N_{\text{area}})</td>
<td>(\text{g m}^{-2})</td>
</tr>
<tr>
<td>Leaf dry mass per area</td>
<td>(LMA)</td>
<td>(\text{g m}^{-2})</td>
</tr>
</tbody>
</table>

*The symbol \(J_{850}\) is used throughout to indicate that \(J_{\text{max}}\) is estimated with a photon flux density of 850 \(\mu\text{mol m}^{-2} \text{ s}^{-1}\).
Table 2.3

Variance components and heritability estimates for primary traits investigated here. The genotypic variance \( (V_G) \), environmental variance \( (V_E) \), residual variance \( (V_R) \), broad-sense heritability \( (H^2) \), and significance \( (p) \) are presented for each of the traits. All variance components were estimated with restricted maximum likelihood. \( V_E \) represents the portion of phenotypic variance attributable to replication blocks and \( V_R \) is the remaining residual variance in the models. Significance values are from likelihood ratio tests comparing models with and without the random effect of ecotype on the traits, where * represents \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \) after correcting the false discovery rate for multiple testing.

<table>
<thead>
<tr>
<th>Trait</th>
<th>( V_G )</th>
<th>( V_E )</th>
<th>( V_R )</th>
<th>( H^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_{sc} )</td>
<td>3.72e-4</td>
<td>7.19e-5</td>
<td>8.44e-4</td>
<td>0.30</td>
<td>*</td>
</tr>
<tr>
<td>( g_m )</td>
<td>0.00733</td>
<td>0.00243</td>
<td>0.0147</td>
<td>0.33</td>
<td>***</td>
</tr>
<tr>
<td>( g_{tot} )</td>
<td>2.35e-5</td>
<td>6.55e-6</td>
<td>5.71e-5</td>
<td>0.29</td>
<td>**</td>
</tr>
<tr>
<td>( J_T )</td>
<td>75.8</td>
<td>57.4</td>
<td>218</td>
<td>0.25</td>
<td>**</td>
</tr>
<tr>
<td>( J_C )</td>
<td>25.8</td>
<td>20.3</td>
<td>88.3</td>
<td>0.22</td>
<td>*</td>
</tr>
<tr>
<td>( J_O )</td>
<td>16.2</td>
<td>9.27</td>
<td>37.5</td>
<td>0.29</td>
<td>**</td>
</tr>
<tr>
<td>( V_{C_{max}} )</td>
<td>21.3</td>
<td>16.7</td>
<td>60.6</td>
<td>0.25</td>
<td>**</td>
</tr>
<tr>
<td>( J_{850} )</td>
<td>148</td>
<td>55.9</td>
<td>228</td>
<td>0.38</td>
<td>***</td>
</tr>
<tr>
<td>( A_N )</td>
<td>0.915</td>
<td>0.552</td>
<td>3.22</td>
<td>0.22</td>
<td>*</td>
</tr>
<tr>
<td>( PR^1 )</td>
<td>0.253</td>
<td>0.145</td>
<td>0.586</td>
<td>0.29</td>
<td>**</td>
</tr>
<tr>
<td>( AN/g_s )</td>
<td>52.7</td>
<td>15.0</td>
<td>55.2</td>
<td>0.48</td>
<td>***</td>
</tr>
<tr>
<td>( \delta^{13}C )</td>
<td>0.930</td>
<td>0.0144</td>
<td>0.291</td>
<td>0.76</td>
<td>***</td>
</tr>
<tr>
<td>( N_{mass} )</td>
<td>0.123</td>
<td>0.00357</td>
<td>0.0504</td>
<td>0.71</td>
<td>***</td>
</tr>
<tr>
<td>( N_{area} )</td>
<td>0.0219</td>
<td>0.00110</td>
<td>0.0116</td>
<td>0.65</td>
<td>***</td>
</tr>
<tr>
<td>( LMA )</td>
<td>5.72</td>
<td>0.450</td>
<td>2.88</td>
<td>0.67</td>
<td>***</td>
</tr>
</tbody>
</table>

\(^1\text{Note that } PR \text{ and } J_O \text{ are direct numeric transformations of one another.}\)
Table 2.4

Pearson product-moment correlation matrix of physiological traits is presented. The phenotypic correlations are presented above the diagonal, and genotypic correlations are below. Genetic correlations were estimated from ecotype best-linear unbiased predictors. Bolded correlations were statistically significant ($p<0.05$) after correcting for multiple testing. Abbreviations are as in Table 2. Note that since $PR$ and $J_O$ are direct numeric transformations of one another they are perfectly correlated.

<table>
<thead>
<tr>
<th></th>
<th>$g_{sc}$</th>
<th>$g_m$</th>
<th>$g_{tot}$</th>
<th>$J_T$</th>
<th>$J_C$</th>
<th>$J_O$</th>
<th>$V_{C_{max}}$</th>
<th>$J_{850}$</th>
<th>$A_N$</th>
<th>$PR$</th>
<th>$A_N/g_s$</th>
<th>$\delta^{13}C$</th>
</tr>
</thead>
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<td>$g_{sc}$</td>
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<td>0.79</td>
<td>0.37</td>
<td>0.49</td>
<td>0.17</td>
<td>0.43</td>
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<td>0.61</td>
<td>0.17</td>
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<td>-0.36</td>
</tr>
<tr>
<td>$g_m$</td>
<td>0.43</td>
<td>---</td>
<td>0.92</td>
<td>0.67</td>
<td>0.81</td>
<td>0.40</td>
<td>0.76</td>
<td>0.71</td>
<td>0.91</td>
<td>0.40</td>
<td>0.15</td>
<td>0.28</td>
</tr>
<tr>
<td>$g_{tot}$</td>
<td>0.77</td>
<td>0.91</td>
<td>---</td>
<td>0.63</td>
<td>0.78</td>
<td>0.35</td>
<td>0.73</td>
<td>0.60</td>
<td>0.91</td>
<td>0.35</td>
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<tr>
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<td>---</td>
<td>0.97</td>
<td>0.94</td>
<td>0.72</td>
<td>0.64</td>
<td>0.86</td>
<td>0.94</td>
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<td>0.62</td>
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<td>---</td>
<td>0.83</td>
<td>0.79</td>
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<td>0.94</td>
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<td>---</td>
<td>0.53</td>
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<td>0.64</td>
<td>1.0</td>
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<tr>
<td>$V_{C_{max}}$</td>
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<td>0.82</td>
<td>0.70</td>
<td>0.65</td>
<td>0.77</td>
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<td>0.57</td>
<td>0.64</td>
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<td>0.08</td>
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<tr>
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<td>0.82</td>
<td>---</td>
<td>0.67</td>
</tr>
<tr>
<td>$\delta^{13}C$</td>
<td>-0.71</td>
<td>0.31</td>
<td>-0.10</td>
<td>0.69</td>
<td>0.58</td>
<td>0.76</td>
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<td>0.31</td>
<td>0.76</td>
<td>0.97</td>
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</tbody>
</table>
Figure 2.1 Relationship between net CO₂ assimilation ($A_n$) and (A) conductance of CO₂, and (B) electron transport. Conductance of CO₂ is presented as stomatal ($g_{sc}$; gold symbols and fit lines), mesophyll ($g_m$; red), and total conductance from the boundary layer to chloroplasts ($g_{tot}$; green). Electron transport is presented as total calibrated-linear ($J_T$; red), and as the partition of electrons utilized as reductant in carboxylation reactions ($J_C$; green). Phenotypic ($r_p$) and genetic ($r_g$) correlations, where significant ($p<0.01$) are presented; (ns) is not-significant. Each ecotype is represented by a unique symbol. Lines are mixed-model fits with ecotype identity and replication-block treated as random effects.
Figure 2.2 The relationship between (A) electron transport driving carboxylation ($J_C$) with stomatal ($g_{sc}$; gold) and mesophyll ($g_m$; red) conductance to CO$_2$, and (B) maximum electron transport ($J_{850}$) and carboxylation ($V_{Cmax}$) capacities. Each ecotype is designated by a unique open symbol. Closed symbols (black in A; red in B) represent ecotype best linear unbiased predictors. Lines are mixed-model fits to the bivariate relationships with ecotype identity and replication as random effects. When significant, phenotypic ($r_p$) and genetic ($r_g$) correlations are presented ($p<0.01$); (ns) is not-significant.
Figure 2.3 Photorespiratory CO₂ efflux ($PR$) as a function of (A) net assimilation ($A_N$), (B) the maximum carboxylation capacity ($V_{Cmax}$), and (C) maximum electron transport capacity ($J_{550}$). Phenotypic correlations ($r_p$) are presented ($p<0.001$); no genetic correlations were significant after correcting for multiple testing. Ecotypes are represented by unique symbols and ecotype means are over-plotted as red squares. Fit lines are drawn from mixed-models with ecotype and replication block as random effects.
Figure 2.4 Leaf-level integrated water-use-efficiency ($\delta^{13}$C) by (A) intrinsic water use efficiency ($A_v/g_s$) and (B) the ratio of intercellular to atmospheric [CO$_2$] ($C_i/C_a$). Phenotypic and genetic correlations were significant ($p<0.001$). Each ecotype is represented by a unique black symbol, and ecotype means are over-plotted as red symbols. Fit lines represent a mixed model fits with replication and ecotype as random effects.
Figure 2.5 Relationships between water use efficiency metrics and components effecting water use efficiency. Correlations between intrinsic water use efficiency ($A_N/g_s$) and (A) $A_N$, (B) stomatal conductance to water vapor ($g_s$), and (C) mesophyll conductance ($g_m$). Integrated water use efficiency ($\delta^{13}C$) is likewise correlated with (D) $A_N$, (E) $g_s$, and (F) $g_m$. Ecotypes are represented by unique symbols and are colored according to their life history strategy with spring annuals in brown and winter annuals in green. Fit lines, presented when significant ($p<0.05$), are linear regressions for spring (brown) or winter (green) subsets of the data.
Figure 2.6 Comparisons of trait values in ecotypes exhibiting spring or winter annual life-history strategies. The traits are (A) nitrogen content per unit leaf area (N_{area}), (B) maximum carboxylation capacity (V_{C_{max}}), (C) maximum electron transport capacity (J_{850}), (D) intrinsic water-use-efficiency (A_{W/g_s}), (E) integrated water use efficiency (δ^{13}C), and (F) leaf mass per area (LMA). Winter annuals (n=6) have significantly (p<0.01) greater trait values than spring annuals (n=8) following unequal-variance t-tests. Eight of the 14 ecotypes exhibit the spring habit. Bar width is scaled to the relative sample size in each group (N=46 for spring, N=34 for winter).
Chapter 3. Mesophyll Conductance Among Soybean Cultivars Sets a Tradeoff Between Photosynthesis and Water-Use

Chapter 3 was accepted for publication in Plant Physiology (doi:10.1104/pp.16.01940) and is presented here as a preprint version formatted to the journal’s specifications.

Abstract

Photosynthetic efficiency is a critical determinant of crop yield potential, though it remains below the theoretical optimum in modern crop varieties. Enhancing mesophyll conductance, i.e. the rate of carbon dioxide diffusion from substomatal cavities to the sites of carboxylation, may increase photosynthetic and water use efficiencies. To improve water-use-efficiency mesophyll conductance should be increased without concomitantly increasing stomatal conductance. Here we partition variance in mesophyll conductance to within and among cultivar components across soybeans grown under both controlled and field conditions, and examine the covariation of mesophyll conductance with photosynthetic rate, stomatal conductance, water-use-efficiency and leaf mass per area. We demonstrate that mesophyll conductance varies more than 2-fold and that 38% of this variation is due to cultivar identity. As expected mesophyll conductance is positively correlated with photosynthetic rates. However, a strong positive correlation between mesophyll and stomatal conductance among cultivars apparently impedes positive scaling between mesophyll conductance and water-use-efficiency in soybean. Contrary to expectations, photosynthetic rates and mesophyll conductance both increased with increasing leaf mass per area. The presence of genetic variation for mesophyll conductance suggests there is potential to increase photosynthesis and mesophyll conductance by selecting for greater leaf mass per area.
Increasing water-use-efficiency though, is unlikely unless there is simultaneous stabilizing selection on stomatal conductance.

**Introduction**

Historical increases in crop productivity are primarily attributable to the optimization of two, out of the four parameters contributing to yield potential. Yield potentials are a function of incoming solar radiation, the interception efficiency of that radiation by the canopy, the efficiency of converting intercepted radiation into biomass, and the proportion of biomass partitioned to harvestable product (i.e., harvest index) (Monteith, 1977). Beginning with the green revolution, major advances in crop yield potential have been realized through maximizing canopy radiation-interception efficiency (Evans, 1993) and harvest indices (Hay, 1995). In soybean, interception efficiency has increased through a combination of later maturation leading to longer growing seasons and a decreased susceptibility to lodging (Koester et al., 2014). Likewise, the harvest index is optimized in many modern crops, including soybean, and regularly accounts for 50% or more of aboveground biomass (Hay, 1995). The optimization of harvest indices and interception efficiencies in many of the most widely cultivated crops is nearing its upper limit (Zhu et al., 2010). However, we can improve upon the remaining determinant of yield potential: the efficiency of converting absorbed light to biomass (Beadle and Long, 1985; Slattery et al., 2013; Koester et al., 2014; Slattery and Ort, 2015; Koester et al., 2016).

Because photosynthesis is the primary determinant of conversion efficiency, several routes to improving crop photosynthetic rates have been identified (for review see Long et al., 2006; von Caemmerer and Evans, 2010; Zhu et al., 2010; Evans, 2013; Ort et al., 2015). Some strategies rely on biological engineering of photosynthetic enzymes – for
example altering Rubisco to reduce photorespiration or to increase carboxylation (Whitney et al., 2011; Betti et al., 2016; Prins et al., 2016), or up-regulating other rate-limiting Calvin cycle enzymes (Lefebvre et al., 2005; Zhu et al., 2007). Another even more ambitious approach aims to re-engineer the entire photosynthetic pathway by introducing C₄-type carbon-concentrating mechanisms into C₃ crops (Mitchell and Sheehy, 2006; Sage and Sage, 2009; Sage and Zhu, 2011). An alternative, and potentially more readily achievable strategy that could improve photosynthetic rates, and possibly water-use-efficiency, is to enhance the mesophyll conductance to CO₂ (gₘ) (Flexas et al., 2013a). Several analyses show that gₘ can limit photosynthetic rates at magnitudes similar to stomatal conductance (gₛ) (Grassi and Magnani, 2005; Galmés et al., 2013; Tomás et al., 2013) and, simulations indicate that a doubling of gₘ in C₃ crops would result in a nearly 20% boost to photosynthetic rates (Zhu et al., 2010). Mesophyll conductance alters the CO₂ concentration gradient from the substomatal cavity (Cᵢ) to the chloroplast stroma (Cₖ) and is presumed to be independent of water loss from the leaf. So an additional advantage of improving photosynthesis through enhancing gₘ is the potential for concurrent improvement of water-use-efficiency (**WUE**).

The expectation that increasing gₘ will improve intrinsic **WUE** (Aᵦ/gₛ) must be tempered by the possibility of a correlation between gₘ and gₛ (e.g., Flexas et al., 2016). A positive relationship between gₘ and gₛ has been reported in several studies (Barbour et al., 2010; Gu et al., 2012; Flexas et al., 2013a; Flexas et al., 2013b), though two studies in wheat detected no relationship between gₘ and gₛ (Jahan et al., 2014; Barbour et al., 2016). It is not yet clear if the coordination between gₘ and gₛ is associated with independent scaling of both diffusional conductances with the overall physiological activity of leaves, or if there is an underlying mechanistic relationship we have yet to
appreciate (e.g. reliance on the same aquaporins in both the diffusion path of CO$_2$ and the outside-xylem portion of hydraulic flow (Flexas et al., 2013b)). Given the potential for coordination between $g_m$ and $g_s$, the absolute value of $g_m$ may be less of a determinant to improving WUE than is the relative value of $g_m$ to $g_s$, i.e. the ratio $g_m/g_s$. However few studies have investigated intraspecific variation in $g_m$, and fewer still have resolved how $g_m$ and $g_s$ covary.

While theory demonstrates that improving $g_m$ will result in greater photosynthetic rates, the available empirical data on $g_m$ to this point has focused primarily on cross-species comparisons. Surprisingly, less attention has been paid to intraspecific comparisons where genetic variation in $g_m$, should it exist, would provide both the genetic material and a guide to select for a reduced diffusional limitation. Across species $g_m$ varies at least 24-fold in seed plants (Tomás et al., 2013) with the lowest values observed in evergreen trees and shrubs, and upper values in grasses and herbaceous dicots (Flexas et al., 2008). From the reports currently available, it appears that $g_m$ varies within genera and species, though most attention has focused on grasses. In studies of barley and rice, where only four cultivars of each were compared, $g_m$ varied among them by $\sim$30% (Barbour et al., 2010; Adachi et al., 2013). Another study in rice found nearly 60% variation among 11 inbred lines (Gu et al., 2012). In wheat, two-fold variation was found among 10 genotypes (Jahan et al., 2014), and three-fold variation among 150 mapping population lines (Barbour et al., 2016). Clearly genetic variation for $g_m$ exists in the monocots, though in 2014 half of the World’s 10 most planted (as total area) crops were eudicots (FAOSTAT 2016; http://faostat3.fao.org/download/Q/QC/E). Tomatoes and grape are the only eudicot food crops for which $g_m$ has been studied at the intrageneric (Muir et al., 2014; Muir et al., 2017) or intraspecific (Galmés et al., 2011a;
Tomás et al., 2014) levels, and estimates of genetic variation for $g_m$ in these and other eudicot crops are lacking, revealing a substantial knowledge gap.

Mesophyll conductance is an emergent trait influenced by many independent leaf properties, with contribution from both structural and biochemical traits (Flexas et al., 2012). Anatomically, higher $g_m$ is associated with thinner cell walls and greater mesophyll cell surface area exposed to intercellular air spaces per unit leaf area ($S_{mes}$) (Evans et al., 2009; Terashima et al., 2011). Cell wall thickness does influence the resistance of CO$_2$ diffusion into cells, but the small variance in cell wall thickness expected among genotypes of short-lived crop leaves developing under uniform conditions should exert little influence on the variance of $g_m$ (e.g., Giuliani et al., 2013). However, $S_{mes}$ partially determines the number of parallel diffusion paths for CO$_2$ into mesophyll cells and differs among species within genera (Giuliani et al., 2013) and genotypes within species (Galmés et al., 2013), providing a key trait underlying variation in $g_m$. Leaf density ($L_D$) and leaf thickness ($L_T$) combine to determine leaf mass per area ($LMA$). Across species the relationship between $g_m$ and leaf mass per area ($LMA$) is negative (Hassiotou et al., 2009; Niinemets et al., 2009) because species with higher $LMA$ have thicker cell walls and greater cell densities leading to reduced $S_{mes}$. Within species, particularly those with relatively thin leaves such as soybean, only the upper limit of $g_m$ seems controlled by $LMA$ (Flexas et al., 2008), which may reflect an altered $LMA$-$S_{mes}$ relationship at lower values of $LMA$ (e.g., Milla-Moreno et al., 2016). For species with low $LMA$ leaves, at the intraspecific level the components of $LMA$ (i.e., $L_D$ and $L_T$) or $LMA$ itself may provide meaningful predictors of $g_m$ as proxies for $S_{mes}$.

We used soybean ($Glycine max$) as a model eudicot crop to assess how $g_m$ varied across cultivars and covaried with leaf physiological and structural traits associated with
photosynthesis (Table 3.1). We addressed several questions: (1) Does $A_N$ scale with $g_m$ as strongly across cultivars of soybean, a thin leaved eudicot, as reported for other crops and cross-species comparisons? (2) If $g_m$ does scale with $A_N$ then does genetic variation exist for this trait? (3) Is water-use efficiency greater in cultivars with greater $g_m$, or (4) is scaling between $g_m$ and $WUE$ precluded by coordination between $g_m$ and $g_s$? And (5) is $LMA$, or are its components, predictive of $g_m$? We hypothesized that (1) the role of $g_m$ on carbon supply would lead to coordination with $A_N$, (2) any correlation between $g_m$ and $g_s$ results in no detectable relationship between $WUE$ and $g_m$, and (3) leaves with greater $LMA$, thickness, and density would exhibit lower $g_m$. These hypotheses were tested on 12 cultivars of edamame soybeans grown under controlled conditions, and then further examined in eight of those cultivars at three growth stages under field conditions. Analyses in two environments and across three growth stages gave us a robust design with which to assess variation and covariation of $g_m$ among cultivars providing confidence in results that were consistent across these groups.

**Results**

To improve the accuracy of our variable-J estimates of $g_m$ we determined the apparent photorespiratory CO$_2$ compensation point ($C_i^*$) and day respiration rate ($R_d$) for all soybean cultivars. $C_i^*$ and $R_d$ were estimated from Laisk curves on chamber grown plants ($n=5$-$8$, $N=72$). The mean $C_i^*$ was 4.08 ($\pm0.058$ s.e.) Pa CO$_2$ and mean $R_d$ was 0.937 ($\pm0.036$ s.e.) $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$. Both $C_i^*$ and $R_d$ differed among cultivars ($p<0.05$; Table 3.2, Figure 3.S1), though only cultvar-specific $R_d$ vales were used under the presumption that the true photorespiratory compensation point ($\Gamma^*$) should not vary across such closely related plants. A sensitivity analysis examining how variable $\Gamma^*$ alters estimates of genetic variance for $g_m$ among cultivars, revealed that $g_m$ does
depend on $\Gamma^*$, but the effects of $\Gamma^*$ on genetic variance in $g_m$ were minimal (Figure 3.S2). The mean $C^*$ here falls between the value of $\Gamma^*$ from tobacco (Bernacchi et al., 2002) commonly used for soybean (Bernacchi et al., 2005; Rosenthal et al., 2014; Köhler et al., 2016) and a value calculated from soybean Rubisco kinetic properties (Gallé et al., 2013), and it further matches exactly the $C^*$ estimated for soybean with similar methodology (Walker and Ort, 2015). Thus, the mean $C^*$ value was used as a proxy for $\Gamma^*$ in all calculations. Cultivar-specific $R_d$ values were used here and ranged from 0.630 to 1.32 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ (Figure 3.S1). Since these $R_d$ values were estimated from plants grown in a controlled environment only, we performed a sensitivity analysis to assess how variation in $R_d$ would alter estimates of genetic variance for $g_m$ from field grown plants (Figure 3.S3) by recalculating $g_m$ for all field measurements using an unvarying $R_d$ for all cultivars ranging from 0.5-1.5 $\mu$mol m$^{-2}$ s$^{-1}$. Estimates of genetic variance in $g_m$ were nearly unresponsive to this magnitude variation in $R_d$ (Figure 3.S3).

Leaf absorptance at 470 and 665 nm ($\alpha$, Table 3.1) was measured on all plants to constrain the calibration of electron transport rates used in the estimation of $g_m$. Absorptance averaged 0.89 (range 0.82 to 0.96; Figure 3.S4; Tables 3.2 and 3.3). Under both controlled and field conditions, cultivar identity was a significant source of variation in absorptance ($p<0.01$) primarily attributable to one cultivar that also had consistently greater relative chlorophyll content as assessed with a clamp-on chlorophyll meter. Absorptance increased with plant growth stage in the field ($p<0.001$), and overall was greater for field grown plants than for those grown in chambers. Relative chlorophyll content differed among cultivars under both field and controlled environment conditions ($p<0.01$). Nitrogen concentrations were determined for the field grown plants; these did not differ among cultivars on a leaf-area or leaf-mass basis. Nitrogen content per leaf
area increased with growth stage in the field (Table 3.3; $p<0.001$), while nitrogen per leaf mass peaked at the early-reproductive growth stage ($p<0.001$).

*Physiological and structural trait variation among cultivars*

When grown in controlled environment chambers phenotypic variance in leaf structure and physiology varied to similar extents across the 12 cultivars studied. Among cultivar variance was greater for physiological than structural traits (Table 3.2). Calibrated electron transport rates ($J_{\text{cal}}$) ranged 1.6-fold with cultivar explaining 31.0% of the variance. Cultivar identity contributed little to the 2.7-fold variance in stomatal conductance ($g_s$), while mesophyll conductance ranged 2.2-fold with 38.8% of total phenotypic variance in $g_m$ found among cultivars. Differences in $g_m$ led to variation in the CO₂-concentration gradient between $C_i$ and $C_c$ ($C_i-C_c$), where 15.8% of the variance was explained by cultivar. A 1.7-fold range in steady-state assimilation ($A_N$) was observed with 37.8% of the variance among cultivars. Little of the phenotypic variance in structural traits was explained by cultivar (Table 3.2).

Eight of the 12 cultivars where also grown in the field and measured at three growth stages that corresponded to late-vegetative (V4 and V5), early-reproductive (R2-R4), and late-reproductive (R6). Cultivar identity and growth stage both affected phenotypic trait variation in the field (Table 3.3). Cultivar tended to have a greater effect on physiological traits, while growth stage tended to have stronger effects on structural traits (Table 3.3). For instance, among cultivar variance was 13.8% for $J_{\text{cal}}$ and 21.1% for $A_N$; neither trait differed by growth stage (Table 3.3; Figure 3.S5 A and D). Stomatal conductance to water vapor ($g_s$) was highest during vegetative growth in July at 425 ± 18 mmol m$^{-2}$ s$^{-1}$ (mean ± s.e.), then declined through reproductive growth to a low of 236 ± 18.3 mmol m$^{-2}$ s$^{-1}$ during the late-reproductive stage in September (Figure 3.S5 B).
seemingly tracking precipitation (Figure 3.S6). Mesophyll conductance (Figure 3.S5 C) and \( C_t - C_c \) (not shown) behaved similarly; both \( g_m \) and \( C_t - C_c \) were indistinguishable between vegetative and early-reproductive growth, then \( g_m \) declined at the late-reproductive stage resulting in an increase of \( C_t - C_c \) (Table 3.3). Phenotypic variance attributed to cultivar for \( g_s \), \( g_m \), and \( C_t - C_c \), was lower in the field than in the controlled environment (compare Table 3.2 & Table 3.3). Intrinsic \( (A_N/g_s) \) and integrated \( (\delta^{13}C) \) water use efficiencies tracked \( g_s \) throughout the season, i.e. \( A_N/g_s \) steadily increased while \( \delta^{13}C \) became less negative from late-vegetative to late-reproductive growth. None of the variance in \( A_N/g_s \) was contributed by cultivar identity, while a third of the variance in \( \delta^{13}C \) was among cultivars (Table 3.3).

Leaf structural traits differed among cultivars and more strongly between growth stages. Though the youngest fully-expanded top-canopy leaves were always sampled, the leaves sampled at the late-reproductive stage were clearly older and more robust than those sampled earlier in the season. Leaf thickness \( (L_T) \) differed negligibly among cultivars, but \( L_T \) did increase at the late-vegetative growth stage (Table 3.3). Among cultivar variance explained some variation in \( L_D \) and \( LDMC \), though again the largest differences were observed at the late-reproductive growth stage (R6). Environmental variance and development dominated variation in \( LMA \), which increased slightly between vegetative and early-reproductive growth, and 1.8-fold in the late-reproductive stage, with no detectable among cultivar contribution (Table 3.3).

**Trait correlations with \( g_m \)**

In the controlled environment, most physiological traits were correlated with one another, and cultivar identity often explained modest amounts of variance in these relationships (Table 3.4). Steady state photosynthetic rate was closely coupled with \( J_{cal} \)
(\(\Omega_0^2=0.840, \ p<0.001\)), \(g_{s-co2}\) (\(\Omega_0^2=0.594, \ p<0.001\)), and \(g_m\) (\(\Omega_0^2=0.776, \ p<0.001\); Figure 3.1 A-C) with 14.5, 11.3, and 10.4%, respectively, of the variance in these relationships found among cultivars. The two diffusional conductances to \(CO_2\), \(g_m\) and \(g_{s-co2}\), were correlated (\(\Omega_0^2=0.287, \ p<0.001\); Figure 3.1 D) and cultivar identity was responsible for 23.3% of the variation in this relationship. No relationship between intrinsic water-use-efficiency \((A_{v}/g_s)\) and \(g_m\) was detected \((p>0.1\); Figure 3.2 A). We separated the effects of \(g_m\) on \(A_{v}/g_s\) without the confounding correlation of \(g_m\) with \(g_{s-co2}\), by analyzing the relationship between the ratio of \(g_m/g_{s-co2}\) and \(A_{v}/g_s\). There was a strong positive relationship between \(g_m/g_{s-co2}\) and \(A_{v}/g_s\) (\(\Omega_0^2=0.849, \ p<0.001\); Figure 3.2 B) with 19.7% of the variance among cultivars. The correlation between \(g_m/g_{s-co2}\) and \(A_{v}/g_s\) may be spurious because \(g_s\) is in the denominator of both terms, i.e., both \(A_{v}/g_s\) and \(g_m/g_{s-co2}\) declined with increasing \(g_s\) or \(g_{s-co2}\) (Figure 3.2 C and D). However, when controlling for \(g_s\), the partial correlation of \(A_{v}/g_s\) and \(g_m/g_{s-co2}\) was still significant \((r_{xy|z}=0.817, \ p<0.001\). Mesophyll conductance was positively correlated with \(LMA\) (\(\Omega_0^2=0.504, \ p<0.01\); Figure 3.3 C), and not correlated with leaf dry matter content. Of the components of \(LMA\), leaf thickness \((L_T)\) and leaf density \((L_D)\), only \(L_T\) was significantly associated with \(g_m\) (\(\Omega_0^2=0.475, \ p<0.05\); Figure 3.3 A).

In the field, correlations among physiological traits were generally strong and often modified across growth stages. To examine the effects of among cultivar trait variation independent of growth stage, linear mixed effects models were fit treating both the predictor trait and growth stage as fixed effects, and cultivar identity as a random effect. Steady state assimilation was coordinated with \(CO_2\) supply as \(g_{s-co2}\) (\(\Omega_0^2=0.748, \ p<0.001\)), \(g_m\) (\(\Omega_0^2=0.689, \ p<0.001\)), or total \(CO_2\) conductance (\(\Omega_0^2=0.795\); not shown), and
with reductant supply as \( J_{\text{cal}} (\alpha_J^2 = 0.743, p < 0.001) \) (Figure 3.4 A-C). Bivariate relationships of \( g_{\text{co2}} \) and \( J_{\text{cal}} \) with \( A_{\text{Ni}} \) differed among growth stages (\( p < 0.001 \) for each); the relationship between \( A_{\text{Ni}} \) and \( g_m \) was not modified by growth stage. Mesophyll conductance and \( g_{\text{co2}} \) were correlated (\( \alpha_G^2 = 0.611, p < 0.001 \)) and differed by growth stage (\( p < 0.001 \); Figure 3.4 D). Intrinsic water-use-efficiency was not significantly related to \( g_m \), but integrated water-use-efficiency (\( \delta^{13}\text{C} \)) was negatively correlated with \( g_m \) (\( \alpha^2 = 0.780, p < 0.05 \); Figure 3.5 A & C) and 27% of the variance in this relationship was among cultivars (Table 3.4). The ratio of intercellular to ambient [CO\(_2\)] (\( C_i/C_a \)) was negatively correlated with \( A_{\text{Ni}}/g_a \) (\( p < 0.001 \)), but not \( \delta^{13}\text{C} \) (Figure 3.5 B&C), though in both cases growth stage modified the relationships (\( p < 0.001 \)). A strong positive relationship existed between \( A_{\text{Ni}}/g_a \) and \( g_m/g_{\text{co2}} \) (\( \alpha_G^2 = 0.762, p < 0.001 \); Figure 3.57 A). As in the controlled environment, this is due partly to spurious correlation with \( g_a \) in both denominators (Figure 3.57 B-C). After controlling for spurious correlation with a partial correlation analysis the effect size was reduced, but the positive \( A_{\text{Ni}}/g_a* g_m/g_{\text{co2}} \) relationship did remain significant (\( r_{xy|z} = 0.605, p < 0.001 \)).

Trait covariation between structural and physiological traits was generally low resulting from the substantially higher \( LMA \) and \( L_D \) at the late-reproductive stage. Within the late-vegetative and early reproductive growth stages positive relationships were observed between \( g_m \) and \( LMA \) or \( L_D \) (Figure 3.58), leading to marginally significant (\( p < 0.1 \)) relationships for \( g_m \) vs. \( LMA \) and \( g_m \) vs. \( L_D \) across all stages after correcting for multiple comparisons. Mesophyll conductance was not associated with \( L_T \) or \( LDMC \) (Figure 3.58). Development had a strong effect on all of these relationships, i.e. without accounting for growth stage, the correlation of \( g_m \) with these structural traits becomes
negative (positive above) because of a significant decrease in \( g_m \) and increase in \( LMA \), \( L_T \), and \( L_D \) observed at the late-reproductive (R6) stage. As expected, across all three growth stages leaf thickness, \( L_D \), and \( LMA \) were each positively correlated with \( A_n \) \((p<0.01, \text{not shown})\).

**Trait correlations were consistent between experiments**

To test the consistency of key trait rankings between the controlled environment and field experiments we compared standardized (see methods) cultivar-mean \( A_n, J_{cal}, g_m \), and \( g_s \) from the late-vegetative growth stage from the field with those measured in the controlled environment (all chamber plants were measured at the late-vegetative stage). Using Spearman’s rank correlations, \( A_n (r=0.81) \) and \( g_m (r=0.90) \) were consistent between experiments (after adjusting for multiple comparisons, \( p<0.05 \) for both; Figure 3.6). Electron transport \((r=0.67)\) and \( g_s (r=0.60) \) were somewhat consistent between chambers and field environments \((p<0.1; \text{Figure 3.6})\). Regression slopes were also tested for consistency across both environments and growth stages using standardized major axis regression (Table 3.S1). Of the 42 possible comparisons, slopes differed in just nine cases, all of which were comparisons of slopes from chamber grown plants to field grown plants (Table 3.S1), indicating that trait correlations were similar between environments and were especially stable among growth stages in the field.

**Trait coordination**

To better understand multivariate trait coordination we performed principal component analyses with all major traits for both controlled environment and field experiments. Of particular interest were the relationships of intrinsic \((A_n/g_s)\) and integrated \((\delta^{13}C)\) water-use-efficiency with \( g_{\text{veg}2}, g_m \), and \( A_n \), as well as relationships between physiological and structural traits. For the controlled environment experiment
the first two principal components cumulatively explained 76.3% of the variance (53.6 and 22.7% respectively). Physiological traits loaded most heavily with the first principal component (PC1) and structural traits with the second principal component (PC2; Figure 3.7 A). All traits loaded positively with PC1 indicating coordination between greater photosynthetic activity and more structurally robust leaves. On PC2 the structural traits loaded positively while $A_N$, $g_m$, and especially $g_{s-co2}$ had slightly negative loadings suggesting a tradeoff in the positive scaling of structure and physiology. Because $A_N/g_s$ is a direct combination of other traits it was not used in fitting the principal component analysis, but was mapped after fitting (see methods). When mapped, $A_N/g_s$ plotted nearly orthogonal to the $g_{s-co2}$ loading in the trait space of the first two principal components (Figure 3.7 A) indicating a CO$_2$ supply-water loss tradeoff and that under chamber conditions intrinsic WUE was primarily influenced by $g_s$.

In the field experiment the first two principal components accounted for 84.4% of the variation (48.3 and 36.1% respectively). A tradeoff between CO$_2$ supply ($g_{s-co2}$ and $g_m$) and leaf structural robustness and water loss was present on PC1 where structural traits ($LMA$, $LDMC$, $N_{area}$) and $\delta^{13}C$ loaded positively, while $A_N$, $g_m$, and especially $g_{s-co2}$ loaded negatively (Figure 3.7 B). Leaves with greater LMA and higher $LDMC$ had lower $g_{s-co2}$ and in turn greater $\delta^{13}C$. There was positive coordination among all traits except $\delta^{13}C$ along PC2. Intrinsic ($A_N/g_s$) water-use-efficiency mapped to nearly the same location as $\delta^{13}C$, and both were inversely located relative to $A_N$, $g_m$, and especially $g_{s-co2}$ on PC1. Individual observations within each growth stage were scattered widely across PC2. A clear pattern was apparent along PC1 with late-vegetative observations clustering on the negative end (i.e., with greater physiological traits values), early-reproductive around zero, and late-reproductive on the positive end with greater leaf structural robustness.
Important similarities in trait coordination and tradeoffs emerged under both controlled and field environments. Physiological traits associated with carbon uptake (i.e., $A_N$, $J_{ca}$) loaded heavily on one principal component and structural traits or water use (efficiency) traits with the other principal component. In both cases, stomatal and mesophyll conductances had the same directionality of loadings on the first two principal components, but $g_{s\cdot co2}$ loaded more strongly along the second principal component indicating some variance in $g_{s\cdot co2}$ independent of $g_m$ (Figure 3.7 A & B). For both experiments, $A_N$ and $g_m$ were more closely associated with one another than either was with $g_{s\cdot co2}$, and mapping of $A_N/g_s$ was to a greater extent in opposition to $g_{s\cdot co2}$ than in accordance with $A_N$. Thus, in both chamber and field grown plants there was some coordination, and some tradeoff between leaf structure (as LMA or LDMC), and the physiological traits $A_N$ and $g_m$.

**Discussion**

Enhancing mesophyll conductance can improve carbon assimilation, may improve intrinsic water-use-efficiency (Flexas et al., 2013a; Flexas et al., 2016) and yield potential in C$_3$ crops (Slattery et al., 2013). Consistent with some recent studies (Barbour et al., 2010; Galmés et al., 2011b; Gu et al., 2012; Giuliani et al., 2013; Jahan et al., 2014; Barbour et al., 2016), we found strong support for the relationship between $A_N$ and $g_m$. Mesophyll conductance is reported to respond to environmental stimuli and stress at timescales ranging from seconds to seasons (e.g., Bernacchi et al., 2002; Grassi and Magnani, 2005; Galmés et al., 2007; von Caemmerer and Evans, 2015; Sorrentino et al., 2016). Here, the relationship between $A_N$ and $g_m$ among 12 soybean cultivars spanning five maturity groups is consistent between growth chamber and field experiments, and across developmental stages (Figure 3.1 and Figure 3.4). The consistent relative
rankings of cultivars for \( g_m \) and \( A_N \) indicates there is genetic differentiation for \( g_m \) in soybean. The degree to which \( g_m \) is genetically determined remains to be fully quantified (see Barbour et al., 2016), but our results indicate that there is potential for selection on \( g_m \) to improve carbon assimilation.

Our second hypothesis was partially supported. We found no significant relationship between intrinsic water-use-efficiency \( (A_N/g_s) \) and \( g_m \) (Figure 3.2 A and Figure 3.5 A); this is consistent with results in rice (Giuliani et al., 2013) and wheat (Jahan et al., 2014), while a negative correlation was found in well-watered and water-stressed tomatoes (Supplementary Table 1 in Galmés et al., 2011). The disparity between the prevailing assumption that enhancing \( g_m \) will improve \( A_N/g_s \) (Flexas et al., 2013a; Flexas et al., 2016) and data inconsistent with this idea stems from the correlation between \( g_m \) and \( g_s \) reported here (Figure 3.1 and Figure 3.4) and elsewhere (Barbour et al., 2010; Galmés et al., 2011a; Gu et al., 2012; Giuliani et al., 2013). The similarity of responses of \( g_m \) and \( g_s \) to water stress, salt stress, light, and \( CO_2 \) lead to the hypothesis that \( g_m \) and \( g_s \) are inextricably co-regulated (Flexas et al., 2008; Vrábl et al., 2009; Sorrentino et al., 2016). Yet, \( g_m \) and \( g_s \) can vary independently. Tazoe et al., (2011) elegantly demonstrated that \( g_m \) was unchanged while \( g_s \) differed in wild type compared to \textit{ost1 Arabidopsis thaliana} mutants with stomata unresponsive to abscisic acid (ABA) or drought (Mustilli et al., 2002). Likewise, Vrábl et al. (2009) concluded that the link between \( g_m \) and \( g_s \) in wheat is “flexible” since \( g_s \), but not \( g_m \), declined after ABA treatment, though others have observed \( g_s \) and \( g_m \) decline in unison following ABA treatment (Sorrentino et al., 2016). Stomatal conductance is also highly sensitive to the leaf-to-air vapor pressure deficit (VPD), and Warren (2008) reported that \( g_s \) could be altered by varying VPD without affecting \( g_m \). Outside of two studies in wheat (Jahan et al., 2014; Barbour et al., 2016),
either no relationship, or a negative relationship, has been found between \( g_m \) and \( A_{\text{N}}/g_s \). The extent of coordination between \( g_m \) and \( g_s \) seems to vary within and among species, and even between cultivars measured here (Figure 3.S5 B & C). And given the potential for \( g_s \) to respond to environmental stimuli independent of \( g_m \) we speculate that inconsistencies in the \( A_{\text{N}}/g_s \) vs. \( g_m \) relationship among studies may simply arise from variation in measurement conditions, or indicate that these relationships are species or genotype specific (e.g., Perez-Martin et al., 2009). Finally, the tendency for a negative \( A_{\text{N}}/g_s \) vs. \( g_m \) relationship was also apparent among aquaporin transformants expressing altered \( g_m \) (Flexas et al., 2016) raising the possibility that variation in aquaporin expression (Perez-Martin et al., 2014) might explain genotypic, environmental, or developmental differences in \( g_m \) reported here and in the literature.

Do dynamic tradeoffs between \( g_m \) and \( A_{\text{N}}/g_s \) affect water-use-efficiency throughout the growing season? Seasonally integrated water-use-efficiency can be estimated from leaf carbon isotope ratios (\( \delta^{13}\text{C} \))(Farquhar et al., 1989). When stomata close, \( A_{\text{N}}/g_s \) increases and substomatal [CO\(_2\)] (\( C_i \)) decreases relative to atmospheric [CO\(_2\)] (\( C_a \)). Consistently lowered \( C_i \) leads to lower discrimination (\( \Delta \)) against \( ^{13}\text{C} \) by Rubisco. Therefore, \( \Delta \) is proportional to the \( C_i/C_a \) ratio, and \( \delta^{15}\text{C} \) in leaf dry matter is interpreted as resulting from the mean leaf lifetime \( C_i/C_a \) ratio, which can be related to \( A_{\text{N}}/g_s \). Larger values of \( \delta^{13}\text{C} \) can be interpreted as higher integrated WUE as long as the leaf-to-air VPD does not vary among samples (Farquhar et al., 1989). From our gas exchange we see that \( g_m \) and \( g_s-\text{co2} \) were correlated (Figure 3.4), thus we observed both greater \( C_i \) and \( C_c \) with greater \( g_m \) allowing Rubisco to discriminate against \( ^{13}\text{CO}_2 \) to a greater extent yielding more negative \( \delta^{13}\text{C} \) values. Since the \( \delta^{13}\text{C} \) of leaves from the field grown plants
was inversely associated with $g_m$ (Figure 3.5 C) we conclude that cultivars with higher $g_m$ had lower integrated $WUE$. The only other study measuring $\delta^{13}C$ and $g_m$ in an intraspecific crop comparison (Giuliani et al., 2013) found a non-significant, but also negative, relationship between the traits. We recognize that mesophyll conductance itself alters carbon isotope fractionation since it partly determines $C_c$. No direct relationship was seen between $C_i/C_a$ and $\delta^{13}C$, indicating that some variation in $\delta^{13}C$ was unrelated to $WUE$ and likely a result of variation in mesophyll conductance (Seibt et al., 2008).

Can we simultaneously increase assimilation and water efficiency in soybean? Optimizing both $WUE$ and $A_N$ will require selection for a greater $g_m$ to $g_s$-$co2$ ratio and not $g_m$ in isolation (Flexas et al., 2013a). We did find a consistent positive relationship between $A_N/g_s$ and $g_m/g_s$-$co2$, and in the field a positive relationship between $\delta^{13}C$ and $g_m/g_s$-$co2$. Similar positive intraspecific scaling of $A_N/g_s$ and $g_m/g_s$ has been found in tomato (Galmés et al., 2011a), rice (Giuliani et al., 2013), and grape (Tomás et al., 2014). Together these results indicate that $WUE$ could be improved by increasing $g_m$, but only at a common $g_s$. After controlling for $g_s$ in the denominator of both $A_N/g_s$ and $g_m/g_s$-$co2$ using partial correlations, the strength of these relationships was greatly reduced, but still significant (Figure 3.2 and Figure 3.S7). Corroborating evidence from the principal components analyses indicates that the variation in $A_N/g_s$ was driven more by variation in $g_s$ than $A_N$. While the $A_N/g_s$-$g_m/g_s$ correlation is clearly significant with meaningful effect in other studies (e.g., Flexas et al., 2013a), the spurious nature of the relationship in our dataset indicates that care should be taken when interpreting this relationship in future studies. Ultimately our data supports a framework of enhancing $A_N$.
through selection for increased cultivar-mean \( g_m \), but not simultaneous improvements in \( A_N/g_s \).

Despite the increased environmental heterogeneity, the reduction in the number of cultivars, and the inclusion of multiple developmental stages, the results from the field experiment were consistent with the chamber study. With the sole exception of \( g_m/g_s\text{-co2} \), trait values had a smaller minima and greater maxima in the field relative to controlled chambers. The increase in phenotypic variance in the field systematically reduced among-cultivar variance for physiological traits (compare Table 3.2 and Table 3.3), which is consistent with theory and previous reports (e.g., Conner et al., 2003). If trait variance is partitioned for the controlled environment experiment including only the cultivars grown in the field, the among cultivar variance for \( g_m \) drops from 38.8 to 25.7%, and for \( A_N \) from 37.8 to 25.6% - values closer to those from the field experiment (11.6 and 21.1% respectively). Despite low power for detection, cultivar-mean \( A_N \) and \( g_m \) rankings were consistent between controlled environment and field growth conditions (Figure 3.6). Bivariate trait relationships were also quite consistent across experiments. All significant relationships observed in the controlled environment, with the exception of the \( g_m\text{-}L_T \), were also found in the field. Further evidence of bivariate consistency is provided by the abundance of overlap in the slopes of bivariate trait relationships between environments and across growth stages (Table 3.S1). These consistencies highlight the role of genetic control over these traits.

Traits potentially contributing to variation of \( g_m \) are generally time consuming and complex to quantify (e.g. anatomical leaf traits). This recognition provides incentive for finding strong correlates of \( g_m \) that are more tractable, and can be used as proxies for \( g_m \) itself. Because \( g_m \) is an emergent trait we quantified LMA, leaf thickness (\( L_T \)), leaf
density \((L_d)\), and leaf dry matter content \((LDMC)\) to test if these traits would provide predictive power to detect variance in \(g_m\) across cultivars. These traits varied little across cultivars grown in chambers, with only \(L_T\) and \(LMA\) significantly related to \(g_m\), or \(A_N\) for that matter, and the variance in \(g_m\) explained by \(LMA\) was modest \((\Omega^2=0.50)\). This result held in measurements of field grown plants though with a much-reduced share of the variance in \(g_m\) explained (Figure 3.S8). To our knowledge the \(g_m\)-\(LMA\) relationship across crop genotypes has only been reported in two other studies and both report a negative correlation among genotypes (Galmés et al., 2011a; Gu et al., 2012). If the effect of growth stage was not accounted for in our analysis of the field dataset the \(g_m\)-\(LMA\) relationship would also be interpreted as negative, while the true relationship among cultivars in our study was always positive.

When is the \(g_m\)-\(LMA\) relationship positive? Light gradients may explain the positive relationship between these two traits in forest canopies, where for example, \(LMA\) in beech trees scaled positively with both \(g_m\) and photosynthetic capacity (Montpied et al., 2009). A positive association of \(g_m\) with \(LMA\) was also observed in populations of \textit{Populus balsamifera} trees (Soolanayakanahally et al., 2009). Milla-Moreno et al., (2016) extended this work on \textit{P. balsamifera}, revealing a positive relationship between \(LMA\) and the thickness of the palisade mesophyll layer. The surface area of mesophyll cells exposed to intercellular air space, known to scale with \(g_m\), increased with palisade thickness providing a mechanistic link between \(g_m\) and \(LMA\) (Milla-Moreno et al., 2016). Whether the positive scaling in soybean presented here can be explained by a similar mechanism is an open question worth exploring. The reported negative correlation of \(LMA\) and \(g_m\) in Galmés et al. (2011) was likely due to differences between watering treatments where stressed plants had higher \(LMA\) and lower \(g_m\), rather than inherent
differences among genotypes. The contrast between our positive $g_m$-$LMA$ relationship and that found by Gu et al. (2012) is harder to explain since they saw a negative relationship between $LMA$ and $g_m$ in rice regardless of plant water status. A recent study in tomato and tomato wild relatives bridging the gap between these intraspecific studies and broad taxonomic surveys where the $g_m$-$LMA$ relationship is consistently negative, demonstrates that at finer taxonomic scales this relationship is more labile resulting from low coordination between $LMA$ and leaf physiological activity (Muir et al., 2017). In any event, if the observed scaling of $g_m$ with $LMA$ holds it suggests an appealing trait for preliminary selection of candidate soybean genotypes with fast photosynthetic physiologies.

**Conclusions**

To artificially select and improve agronomically relevant traits, genotypic variation must exist for those traits and that variation must be heritable (Falconer and Mackay, 1996). In this study we show that there is genetic variation for $g_m$ and that variation is highly coordinated with leaf photosynthetic physiology, and to a lesser extent with coarse leaf structure across soybean cultivars. Genetic variation for $g_m$ (Jahan et al., 2014) and the recent detection of the only known quantitative trait locus associated with $g_m$ hints at the genetic basis for $g_m$ in wheat (Barbour et al., 2016). Whether QTL can be identified in other taxa remains to be seen. Indeed, the genetic basis, magnitude, and nature of genetic variation in $g_m$ must continue to be evaluated in wheat and other taxa, and in variable environments to fully grasp its potential for improving crop productivity. However in soybean, unlike wheat (e.g., Barbour et al., 2016), the coupling of $g_m$ and $g_s$ may interfere with the potential for improving water-use-efficiency through selection on $g_m$. 
Methods

Controlled environment experiment

Twelve cultivars of soybean (Glycine max) with varying maturity group status were obtained from a number of sources (Table 3.S2) and grown under controlled conditions in growth chambers. Seeds were directly sown in 3.75 L pots containing Pro-Mix HP media (Premier Tech Horticulture, Quakertown, Pennsylvania, USA). Plants were maintained at 25:21°C and 16:8 h, light:dark cycle, with an irradiance of approximately 550 µmol m\(^{-2}\) s\(^{-1}\) at the top of the canopy. All plants were well watered and fertilized weekly with a solution of Plantex (Plant Products Co., Brampton, Ontario, Canada). Pots were rotated within chambers every fourth day and between chambers weekly. Gas exchange was measured before flowering on the middle leaflet of the fourth or fifth trifoliate, whichever was the youngest and fully expanded. The relative chlorophyll content of leaves was estimated with a SPAD chlorophyll meter (SPAD 502 Plus, Spectrum Technologies, Inc., Aurora, IL, USA) prior to measurement and an effort was made to only select leaves within a range of 30-40 relative chlorophyll units. Three leaves were below 30, and one was above 40. Chamber grown plants were used for \(A_N\) and \(C_i\) curves to estimate \(g_m\), and Laisk curves to estimate biochemical properties.

Field experiment

Two plants of each cultivar from the controlled environment experiment were transferred to a glasshouse and grown for seed collection. Plants were well watered, fertilized weekly, and allowed to grow until they naturally senesced. Adequate seed was collected from eight cultivars to allow planting in the field the following summer (Table 3.1). The field plot consisted of 13, 10-meter long rows. Cultivars were planted within rows in one-meter long groupings with 30 cm between groups, and eight groups per row.
The eight cultivars of edamame soybean were confined to four rows, interspersed among the remaining nine rows that were planted with soybeans from other experiments. Seeds were sown to a depth of 3 cm and separated by 7.5 cm on 4 June 2015. The plot is located in a river flood-plain and is underlain by Haymond silt loam soil of alluvial origin (Hay1AO; USDA NRCS). Total precipitation throughout the growing season (June through September) was 432 mm, the mean daily temperature was 21.7°C, and absolute min./max. temperatures were 7.0/33.0°C (Figure 3.S6).

Precipitation immediately preceding and proceeding seed sowing was low (Figure 3.S6). To improve germination and seedling establishment we irrigated the field three times during the week after planting. The field was rainfed thereafter. Gas exchange was measured on plants at three times throughout the season (see next paragraph). Precipitation was high preceding the first gas exchange campaign and relatively lower preceding the second and third (Figure 3.S6).

Leaves were collected for gas exchange measurements in the laboratory at three developmental stages: late-vegetative (plants at V4 and V5), early-reproductive (R2 to R4), and late-reproductive (R6). As is common with soybeans (Bernacchi et al., 2005; Ainsworth et al., 2007), the youngest completely expanded trifoliates were excised predawn in the field and stored in the dark with petioles in water until they could be recut under water in the laboratory. Leaves were removed from the dark 25 minutes before measurement and allowed to acclimate under an LED panel at approximately 1650 µmol m⁻² s⁻¹. Aᵣ-Cᵣ curves were measured on all leaves.

Gas exchange measurements

Determination of R₄ and the CO₂ compensation point — So called “Laisk curves” were measured on plants from growth chambers to calculate the apparent CO₂
compensation point in the absence of mitochondrial respiration ($C_{i^*}$) and the respiration rate in the light ($R_d$) of each cultivar (Laisk, 1977; Brooks and Farquhar, 1985). Leaves were acclimated at 25°C, a CO$_2$ concentration of 400 µmol mol$^{-1}$, a photosynthetic photon flux density (PPFD) of 1200 µmol m$^{-2}$ s$^{-1}$, and a vapor pressure deficit of <1.5, with an open gas exchange system (LI-6400; LiCor Inc., Lincoln, NE) and the 2x3 cm broadleaf chamber (LI-6400-02B). Sub-ambient CO$_2$-response curves (CO$_2$ setpoints of 90, 75, 55, and 42 µmol mol$^{-1}$) were measured at three sub-saturating PPFDs (235, 175, and 125 µmol m$^{-2}$ s$^{-1}$). Leaves were re-acclimated at ambient conditions between curves.

To control for the large differential in CO$_2$ concentrations between the ambient air and the leaf chamber and the low fluxes at sub-ambient [CO$_2$], the entire chamber head was loosely sealed in a plastic bag immediately before and during the response curves – allowing the exhaust air from the leaf chamber to surround the outside of the chamber.

The first CO$_2$ setpoint was not used in analyses since it took nearly that length of time for the air in the bag to turnover completely at the low flow rate (250 µmol s$^{-1}$) used for Laisk curves. Laisk curves were measured on five to eight replicates of each cultivar.

$C_{i^*}$ and $R_d$ were calculated by the modified Slope-Intercept method of Walker and Ort (2015). For each cultivar a linear regression was performed on the aggregated CO$_2$-response curves for a given PPFD. The slopes and intercepts from the three regressions were extracted. A second linear regression was performed with the slopes from the first regressions as the x-values, and intercepts as the y-values. $C_{i^*}$ was taken as the absolute value of the slope from the second regression, while $R_d$ was taken as the y-intercept. We found no differences between $C_{i^*}$ and $R_d$ calculated by the Slope-Intercept method of Walker and Ort and the traditional common-slope method (paired t-tests, $p > 0.1$ for both), or the standard deviations of these estimates. The procedure followed here
for measuring Laisk curves though did follow several of the recommended procedures of Walker and Ort (2015). Measuring only four sub-ambient CO$_2$ partial pressures assured that curves at each irradiance were completed rapidly and minimized deactivation of Rubisco. Maximum CO$_2$ partial pressures used in fittings were also consistently below 10 Pa. Do note that Walker and Ort (2015) further recommend use of at least four irradiances, and additional recommendations have since been proposed (Hanson et al., 2016).

$A_n$-$C_i$ curves

Combined CO$_2$-response and fluorescence curves were measured on five to six replicates of each cultivar for the controlled environment experiment, and on one plant from each row-by-cultivar combination from the field experiment providing four replicates of each cultivar per growth stage (with the exception of one cultivar that had only three replicates per stage due to poor germination). Leaves were clamped into the 6400-40 fluorescence head cuvette and allowed to acclimate to ambient conditions for $\geq$30 minutes. Ambient conditions for $A_n$-$C_i$ curves were equivalent to those for Laisk curves except that a flow rate of 300 $\mu$mol s$^{-1}$ was used, and for leaves from the field PPFD was set to 1800 $\mu$mol m$^{-2}$ s$^{-1}$. Once steady-state conditions were reached a point was logged and the [CO$_2$] was iteratively changed in the sequence 400, 325, 250, 175, 100, 50, 400, 400, 500, 650, 950, 1250, 1600, 2000 $\mu$mol mol$^{-1}$. At each CO$_2$ setpoint gas exchange parameters and steady state fluorescence ($F_s$) were logged and a multiphase flash chlorophyll fluorescence routine was executed following the recommended procedures of Loriaux et al. (2013), to determine the maximum ($F_{m}'$) fluorescence. Following this response curve the leaf was allowed to re-acclimate to ambient conditions until net assimilation and stomatal conductance ($g_s$) returned to steady-state conditions. The air
stream supplied to the leaf was then switched to a humidified tank of N₂ with 1% O₂ and a second CO₂-response curve was executed with only sub-ambient CO₂ concentrations (400, 325, 250, 175, 100, and 50 µmol mol⁻¹). Again, F₆ and F₇ were estimated with the multiphase flash routine at each setpoint.

Diffusional leaks during CO₂-response curves resulting from the large differentials in [CO₂] between the inside of the leaf chamber and the ambient air can cause substantial error in leaf flux rates (Flexas et al. 2007; Rodeghiero et al. 2007). We estimated diffusional leaks by measuring identical curves on heat-inactivated leaves (n=12) according to Flexas et al. (2007). The apparent photosynthetic rate of heat-inactivated leaves was subtracted from the photosynthetic rate of experimental leaves, followed by correction of Cᵢ following the equations used by the LI-6400 (Flexas et al., 2007; 2012).

**Variable-J gₘ calculation**

Several methods exist to estimate gₘ and their relative strengths and weaknesses are reviewed elsewhere (Pons et al., 2009; Gu and Sun, 2014). Here we employed methods and protocols to minimize errors that can arise using the variable-J method (Pons et al., 2009). Mesophyll conductance was estimated with the variable-J equation of Harley et al., (1992):

$$ g_m = \frac{A_N}{C_i - \frac{\Gamma^* (J + 8(A_N + R_d))}{J - 4(A_N + R_d)}} $$

Equation 3.1

where, Aₙ and Cᵢ were the leak corrected gas exchange values from the LI-6400, Rₙ values were cultivar specific values taken from Laisk curves, and Γ* was approximated by the mean of all Cᵢ* values also obtained from Laisk curves (see above or below). The electron transport rate (J) was was estimated as follows. First the quantum yield of photosystem II (Φₚₛᵲᵲ) and the quantum yield of CO₂ fixation (Φₚₒ₂) were quantified as:
\[ \Phi_{PSII} = \frac{(F_m' - F_s')}{F_m'} \quad \text{Equation 3.2} \]
\[ \Phi_{CO2} = \frac{(A_N + R_d)}{(aPPFD)} \quad \text{Equation 3.3} \]

where \( \alpha \) was the leaf absorptance measured at 470±5 and 665±5 nm (the peak wavelengths emitted by the 6400-40 light source) immediately following gas exchange using a spectroradiometer and leaf-clamp integrating sphere (Jaz Spectroclip, Ocean Optics, Inc., Dundee, Fl.). Absorptance at the two wavelengths was weighted to account for the gas-exchange measurement light being 10% blue and 90% red. The average of three measures of absorptance made across the leaf blade, avoiding major veins, were used in calculations. Using the CO\(_2\)-response curve measured at 1% O\(_2\), a linear regression of \( \Phi_{PSII} \) on \( \Phi_{CO2} \) was performed and the regression coefficients were then used to calibrate the \( \Phi_{PSII} \) values at 21% O\(_2\) (Valentini et al., 1995; Long and Bernacchi, 2003) as:

At 1% O\(_2\):
\[ \Phi_{PSII} = k\Phi_{CO2} + b \quad \text{Equation 3.4} \]

At 21% O\(_2\):
\[ \Phi_{CAL} = 4(\Phi_{PSII} - b)/k \quad \text{Equation 3.5} \]

A calibrated electron transport rate (\( J_{cal} \)) was then calculated as:
\[ J_{cal} = \Phi_{CAL}PPFD \quad \text{Equation 3.6} \]

and used in the variable-J equation for estimating \( g_m \). CO\(_2\)-response curves were measured at 1% O\(_2\), and the above electron transport rate corrections performed, for all plants excepting the field sampling at the early reproductive stage. In order to facilitate more rapid sampling at this stage, thus standardizing maturity as much as was possible, the 1% O\(_2\) curves on these plants were omitted. The mean slope and intercept values from equation 4 resulting from measurements at the late-vegetative stage were used in this instance to correct the electron transport rate following equations 5 and 6, with the addition of PPFD being corrected with measured \( \alpha \) values.
Values of $g_m$ reported throughout the manuscript are those calculated from measurements where the ambient [CO$_2$] ($C_a$) in the reference air stream was 325 µmol mol$^{-1}$. Estimating $g_m$ with a $C_a$ of 250, 325, and 400 µmol mol$^{-1}$, resulted in an inverse relationship between $g_m$ and $C_a$. The correlation of $g_m$ calculated at each of these three $C_a$ values was high ($r>0.9$, $p<0.001$), and the mean of the three estimates was not significantly different from the value calculated at $C_a$=325. These values were ultimately used as we assume a larger drop in [CO$_2$] across the boundary layer of leaves outside of the well-mixed gas exchange chamber, and therefore the $g_m$ estimates at $C_a$=325 are likely more representative of the average values under growth conditions.

The CO$_2$ concentration in the chloroplast ($C_c$) was calculated with the estimated $g_m$ values according to Fick’s first law:

$$C_c = C_i - \frac{A_N}{g_m}$$

Equation 3.7

and used to calculate the CO$_2$ concentration gradient from $C_i$ to $C_c$ ($C_i-C_c$), and the ratios of $C_i/C_a$ and $C_c/C_i$.

**Leaf morphology**

After gas exchange and absorptance measurements, seven 1-cm diameter punches were taken from the leaf lamina avoiding primary veins. Lamina thickness was measured on four punches with digital calipers and averaged to determine leaf thickness ($L_T$). The punches were then weighed for fresh mass, dried for >72 hours at 65°C, and weighed again for dry mass. Leaf dry mass per area (LMA) was calculated from dry mass and the cumulative area of the punches. Dividing LMA by $L_T$ yielded leaf density (LD). Then leaf dry matter content (LDMC) was calculated as the ratio of dry to fresh mass. After massing, the dry leaf tissue from plants in the field experiment was ground to a fine
powder and analyzed for carbon, nitrogen, and carbon-13 content (δ^{13}C) at The University of Illinois.

Statistical analysis

All analyses and visualizations were performed in R v3.3.0 (R Core Team, 2015). To estimate the influence of genetics on cultivar trait differentiation, variation among cultivars for traits of interest from the controlled environment experiment was assessed by partitioning total phenotypic variance within and among cultivars with restricted maximum likelihood (REML). For field grown plants all cultivars were replicated in four blocks within a larger soybean field. Cultivar and block were treated as random effects with growth stage as a fixed effect using the lmer function of the lme4 package (Bates et al., 2015). Then the genetic component of phenotypic variance was partitioned using REML. Bivariate trait relationships were analyzed with linear mixed-effects models where one of the traits was treated as a fixed effect predictor, block and cultivar as random effects, also with lmer. For mixed-effects models the significance of fixed effects were determined with t-tests using Satterthwaite-approximated degrees of freedom implemented with the lmerTest package (Kuznetsova et al., 2016). Significance of random effects were determined with likelihood ratio tests comparing models with and without the random effect. Effect sizes for bivariate relationships are reported as Ω^2 values, a mixed model analog to $R^2$ calculated as one minus the variance in residuals divided by the variance in the fitted response variable (Xu, 2003). The significance level of all tests was adjusted to reflect multiple-testing on non-independent observation by controlling for the false-discovery-rate and adjusting p-values accordingly (Benjamini and Hochberg, 1995).
To test for univariate cultivar trait consistencies across experiments we compared values of key traits ($A_N$, $g_s$, $g_m$, and $J_{cal}$) as measured in the controlled environment (measured during vegetative growth) to the late-vegetative stage from the field experiment for all cultivars measured in both experiments. We divided cultivar-mean trait values by the maximum cultivar-mean value in each experiment to produce standardized proportional rankings. Then Spearman’s rank correlations were performed on the standardized traits between experiments using a one-tailed test to determine if the relative rankings of cultivars were consistent in chamber and field grown plants. To examine if bivariate relationships were consistent across experiments we compared the slopes of the primary relationships from the controlled environment experiment and all three growth-stages from the field experiment with standardized major-axis regression using the smatr package (Warton et al., 2012). Slopes were considered different at $\alpha=0.05$ after correcting for multiple comparisons.

Multivariate trait coordination was assessed with principal components analysis (PCA). Of all the traits measured in the controlled environment experiment we included $A_N$, $g_{s\cdot co2}$, $g_m$, $J_{cal}$, LMA, and LDMC as primary variables in fitting the analysis. Since the calculation of $A_N/g_s$ is a linear combination of other variables, it was included as a supplementary variable that was mapped in the principal component trait space, but not used in the fitting. For the field experiment we included $A_N$, $g_{s\cdot co2}$, $g_m$, $J_{cal}$, $N_{area}$, $\delta^{13}C$, LMA, and LDMC. In addition to again mapping $A_N/g_s$ as a supplementary continuous variable, growth stage was included as a supplementary factor variable to investigate any seasonal shifts in the positioning of observations. PCAs were fit with the FactoMineR package (Lê et al., 2008).
All data for reproducing the figures and analyses in this manuscript are available from the Dryad Digital Repository: doi:10.5061/dryad.2gd3b

References


Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon Isotope Descrimination and


signalling are predominantly governed by modifications of mesophyll conductance for CO2. Plant, Cell Environ 36: 542–552


Kuznetsova A, Brockhoff PB, Christensen RHB (2016) lmerTest: Tests in Linear Mixed Models


Milla-Moreno EA, McKown AD, Guy RD, Soolanayakanahally RY (2016) Leaf mass area predicts palisade structural properties linked to mesophyll conductance in balsam
poplar. Botany 94: 225–239


and scaling up by models. J Exp Bot 64: 2269–2281


Table 3.1

List of all traits estimated in the study, the symbols used throughout the text, and their units.

<table>
<thead>
<tr>
<th>Category and Trait</th>
<th>Symbol</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apparent photorespiratory CO\textsubscript{2} compensation</td>
<td>$C_i^*$</td>
<td>Pa</td>
</tr>
<tr>
<td>Mitochondrial respiration in the light</td>
<td>$R_d$</td>
<td>$\mu$mol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>Absorptance at 470 and 665 nm</td>
<td>$\alpha$</td>
<td>unitless</td>
</tr>
<tr>
<td>Chlorophyll content</td>
<td>SPAD</td>
<td>unitless</td>
</tr>
<tr>
<td>Net assimilation rate</td>
<td>$A_N$</td>
<td>$\mu$mol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>Maximum light &amp; CO\textsubscript{2} saturated assimilation rate</td>
<td>$A_{\text{max}}$</td>
<td>$\mu$mol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>Stomatal conductance to water vapor</td>
<td>$g_s$</td>
<td>mol H\textsubscript{2}O m\textsuperscript{-2} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>Stomatal conductance to CO\textsubscript{2}</td>
<td>$g_{\text{co}2}$</td>
<td>mol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>Mesophyll conductance</td>
<td>$g_m$</td>
<td>$\mu$mol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1} Pa\textsuperscript{-1}</td>
</tr>
<tr>
<td>Ratio of mesophyll to stomatal conductance</td>
<td>$g_m/g_{\text{co}2}$</td>
<td>mol CO\textsubscript{2} mol\textsuperscript{-1} CO\textsubscript{2}</td>
</tr>
<tr>
<td>Intrinsic water use efficiency</td>
<td>$A_N/g_s$</td>
<td>$\mu$mol CO\textsubscript{2} mol\textsuperscript{-1} H\textsubscript{2}O</td>
</tr>
<tr>
<td>Integrated water use efficiency</td>
<td>$\delta^{13}$C</td>
<td>%</td>
</tr>
<tr>
<td>Calibrated linear electron transport rate</td>
<td>$J_{\text{cal}}$</td>
<td>$\mu$mol electrons m\textsuperscript{-2} s\textsuperscript{-1}</td>
</tr>
<tr>
<td>CO\textsubscript{2} concentration gradient between intercellular airspaces and chloroplast stroma</td>
<td>$C_i-C_c$</td>
<td>$\mu$mol CO\textsubscript{2} mol\textsuperscript{-1} air</td>
</tr>
<tr>
<td>Nitrogen concentration</td>
<td>$N_{\text{mass}}$</td>
<td>%</td>
</tr>
<tr>
<td>Nitrogen concentration per area</td>
<td>$N_{\text{area}}$</td>
<td>mg N m\textsuperscript{-2} leaf</td>
</tr>
<tr>
<td><strong>Leaf structure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf thickness</td>
<td>$L_T$</td>
<td>mm</td>
</tr>
<tr>
<td>Leaf density</td>
<td>$L_D$</td>
<td>g cm\textsuperscript{-3}</td>
</tr>
<tr>
<td>Leaf mass per area</td>
<td>$LMA$</td>
<td>g m\textsuperscript{-2}</td>
</tr>
<tr>
<td>Leaf dry matter content</td>
<td>$LDMC$</td>
<td>g dry g\textsuperscript{-1} fresh</td>
</tr>
</tbody>
</table>
Variance partitioning to within and between cultivars for traits measured as part of the controlled environment experiment, and those used for both experiments (i.e. $C_i^*$ and $R_d$). Variance attributed to cultivar provides an upper-limit estimate of the contribution of genetics to observed trait variability. Trait abbreviations are as in Table 2. Min and Max are the minimum and maximum trait values. The variance components were estimated by partitioning variance within and between cultivars with restricted maximum likelihood (REML).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Range</th>
<th>Variance components</th>
<th>Variance attributed to cultivar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Cultivar</td>
</tr>
<tr>
<td>$C_i^*$</td>
<td>3.07</td>
<td>5.60</td>
<td>4.172x10^{-2}</td>
</tr>
<tr>
<td>$R_d$</td>
<td>0.327</td>
<td>1.57</td>
<td>1.904x10^{-2}</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>0.843</td>
<td>0.917</td>
<td>8.876x10^{-5}</td>
</tr>
<tr>
<td>SPAD</td>
<td>28.0</td>
<td>41.5</td>
<td>1.909</td>
</tr>
<tr>
<td>$A_N$</td>
<td>15.9</td>
<td>27.8</td>
<td>2.905</td>
</tr>
<tr>
<td>$A_{\text{max}}$</td>
<td>19.5</td>
<td>38.9</td>
<td>6.113</td>
</tr>
<tr>
<td>$g_s$</td>
<td>0.155</td>
<td>0.421</td>
<td>6.174x10^{-4}</td>
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<tr>
<td>$g_{\text{bc}}$</td>
<td>0.0966</td>
<td>0.263</td>
<td></td>
</tr>
<tr>
<td>$g_n$</td>
<td>1.22</td>
<td>2.71</td>
<td>4.224x10^{-2}</td>
</tr>
<tr>
<td>$g_{\text{bc}}$</td>
<td>0.410</td>
<td>1.21</td>
<td>0</td>
</tr>
<tr>
<td>$C_i^*-C_c$</td>
<td>90.2</td>
<td>144</td>
<td>18.33</td>
</tr>
<tr>
<td>$A_N/g_s$</td>
<td>55.1</td>
<td>109</td>
<td>0</td>
</tr>
<tr>
<td>$J_{\text{cal}}$</td>
<td>135</td>
<td>219</td>
<td>127.2</td>
</tr>
<tr>
<td>$L_T$</td>
<td>0.163</td>
<td>0.285</td>
<td>1.025x10^{-4}</td>
</tr>
<tr>
<td>$L_D$</td>
<td>0.0771</td>
<td>0.181</td>
<td>1.035x10^{-4}</td>
</tr>
<tr>
<td>LMA</td>
<td>20.1</td>
<td>39.1</td>
<td>1.113x10^{-16}</td>
</tr>
<tr>
<td>LDMC</td>
<td>0.163</td>
<td>0.241</td>
<td>4.402x10^{-5}</td>
</tr>
</tbody>
</table>
Anova statistics from linear mixed effects models for traits measured in the field experiment. For each trait the growth stage of measurement was treated as a fixed effect, and cultivar identity as a random effect to partition phenotypic variance to within and between cultivars. Trait abbreviations are as in Table 2. Min and Max are the minimum and maximum trait values. Model degrees of freedom are type III Satterthwaite approximates. Variance components were estimated by partitioning variance within and between cultivars with REML. Significance levels are: n.s. for \( p > 0.05 \), and *** for \( p < 0.001 \) after adjusting for multiple comparisons. Effects were not estimated for \( g_{s-co2} \) since it is a direct transformation of \( g_s \).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Range</th>
<th>Growth Stage, Fixed Effect</th>
<th>Variance Component</th>
<th>Variance attributed to cultivar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>( F_{2,78-86} )</td>
<td>( p )</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>0.815</td>
<td>0.955</td>
<td>59.5</td>
<td>***</td>
</tr>
<tr>
<td>SPAD</td>
<td>25</td>
<td>54.8</td>
<td>129.1</td>
<td>***</td>
</tr>
<tr>
<td>( A_N )</td>
<td>7.8</td>
<td>33.4</td>
<td>3.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>( g_s )</td>
<td>0.0988</td>
<td>0.585</td>
<td>35.2</td>
<td>***</td>
</tr>
<tr>
<td>( g_{s-co2} )</td>
<td>0.0618</td>
<td>0.365</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( g_m )</td>
<td>0.39</td>
<td>2.42</td>
<td>8.7</td>
<td>***</td>
</tr>
<tr>
<td>( g_m/g_{s-co2} )</td>
<td>0.445</td>
<td>1.389</td>
<td>33.4</td>
<td>***</td>
</tr>
<tr>
<td>( C_T/C_c )</td>
<td>62.0</td>
<td>229.8</td>
<td>14.0</td>
<td>***</td>
</tr>
<tr>
<td>( A_B/g_s )</td>
<td>41.5</td>
<td>114.0</td>
<td>67.1</td>
<td>***</td>
</tr>
<tr>
<td>( \delta^{13}C )</td>
<td>-31.31</td>
<td>-26.2</td>
<td>107.8</td>
<td>***</td>
</tr>
<tr>
<td>( J_{cal} )</td>
<td>95.0</td>
<td>300.4</td>
<td>1.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>( N_{area} )</td>
<td>0.380</td>
<td>3.163</td>
<td>55.5</td>
<td>***</td>
</tr>
<tr>
<td>( N_{mass} )</td>
<td>1.937</td>
<td>5.891</td>
<td>19.9</td>
<td>***</td>
</tr>
<tr>
<td>( L_T )</td>
<td>0.145</td>
<td>0.3025</td>
<td>39.8</td>
<td>***</td>
</tr>
<tr>
<td>( L_D )</td>
<td>0.105</td>
<td>0.281</td>
<td>184.2</td>
<td>***</td>
</tr>
<tr>
<td>( LMA )</td>
<td>19.6</td>
<td>80.8</td>
<td>147.4</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>LDMC</td>
<td>0.172</td>
<td>0.374</td>
<td>244.6</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
</tbody>
</table>

Table 3.4

Estimates of cultivar effect on bivariate relationships for the controlled environment and field experiments. Variance components were estimated with REML. Significance of the cultivar effect was determined with likelihood ratio tests and \( p \)-values were adjusted for multiple testing. Significance levels are: n.s. for \( p > 0.05 \), * for \( p < 0.05 \), and ** for \( p < 0.01 \). Abbreviations are as in Table 3.2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Trait1</th>
<th>Trait2</th>
<th>Variance explained by cultivar (%)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlled environment</td>
<td>( A_N )</td>
<td>( g_s )</td>
<td>11.3</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>( A_N )</td>
<td>( g_m )</td>
<td>10.4</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>( A_N )</td>
<td>( J_{cal} )</td>
<td>14.5</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>( g_m )</td>
<td>( g_{so2} )</td>
<td>23.3</td>
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Figure 3.1 Relationships between light saturated steady-state net assimilation ($A_N$) and (A) total calibrated electron transport ($J_{cal}$), (B) stomatal conductance to CO$_2$ ($g_{s-co2}$), (C) mesophyll conductance ($g_m$), and (D) $g_{s-co2}$ and $g_m$ for the 12 soybean cultivars grown in the controlled environment experiment. Symbols are different cultivars indicated by the legend, $n=5$-6 replicates. Regression lines and coefficients of determination ($\Omega^2$) are from linear mixed effects models (in all cases $p<0.01$).
Figure 3.2 Relationships between intrinsic water-use-efficiency ($A_N/g_s$) and (A) mesophyll conductance ($g_m$), (B) the ratio of mesophyll to stomatal conductance to CO$_2$ ($g_m/g_{s-co2}$), (C) $g_{s-co2}$, and (D) between $g_m/g_{s-co2}$ and $g_{s-co2}$ for the 12 soybean cultivars in the controlled environment experiment. Different symbols represent the different cultivars indicated in the legend, and $n=5$-6. Regression lines, coefficients of determination ($R^2$), and $p$-values are from linear mixed models. In B the partial correlation ($r_{xyz}$) of $A_N/g_s$ with $g_m/g_{s-co2}$ after accounting for $g_s$ is also presented.
Figure 3.3 The relationships between mesophyll conductance \( (g_m) \) and leaf structural traits: (A) leaf mass per area \( (LMA) \), (B) leaf thickness \( (L_T) \), (C) leaf density \( (L_D) \), and (D) leaf dry matter content \( (LDMC) \) for chamber grown soybeans from the controlled environment experiment. Different symbols are indicate different cultivars, \( n=5-6 \).

Regression lines, coefficients of determination \( (R^2) \), and \( p \)-values are from linear mixed models with cultivar as a random effect.
Figure 3.4 Relationship between (A) steady-state net assimilation rate ($A_N$) and total calibrated electron transport ($J_{cal}$), $A_N$ and stomatal conductance to CO$_2$ ($g_{s-co2}$), (C) $A_N$ and mesophyll conductance ($g_m$), and (D) $g_{s-co2}$ and $g_m$. Data are from the eight soybean cultivars in the field experiment and measured at the late-vegetative (V5-V6, black), early-reproductive (R2-R4, dark grey), and late-reproductive (R6, light grey) growth stages. Different symbols represent different cultivars, with $n=4$ replicates for each cultivar-by-growth stage combination, except GS22 where $n=3$. Coefficients of determination ($\Omega^2$) and regression lines are from linear mixed models with the x-variable and growth stage as predictors, and cultivar treated as a random effect ($p<0.001$ for all).
Figure 3.5 Relationships between intrinsic water-use-efficiency ($A_{n}/g_{s}$) and (A) mesophyll conductance ($g_{m}$) and (B) the ratio of intercellular to ambient CO$_{2}$ concentrations ($C_{i}/C_{a}$), and the relationships between integrated water use efficiency ($\delta^{13}$C) and (C) $g_{m}$ and (D) $C_{i}/C_{a}$ from the field experiment. Different symbols represent different cultivars (see Figure 4 legend) with $n=4$ replicates, and shading represent late-vegetative (V4-V5 black), early-reproductive (R2-R4, dark grey), and late-reproductive (R6, light grey) growth stages. The dashed line is a marginally significant ($p<0.1$), while the solid lines are significant ($p<0.05$) according to linear mixed models with growth stage and the variable on the x-axis as fixed effect predictors and cultivar identity as a random effect.
In all cases growth stage was a significant predictor ($p<0.001$). Cultivar identity significantly improved model fit in C and D ($p<0.001$).
Figure 3.6 Consistency among estimates of light saturated assimilation ($A_n$), stomatal conductance to water vapor ($g_s$), mesophyll conductance ($g_m$), and calibrated total electron transport ($J_{cal}$) for late-vegetative (V2-V4) growth stage soybean plants grown in a controlled environment or in the field. Symbols are cultivar-mean values standardized as a proportion of maximum cultivar-mean trait values. Correlations and $p$-values are Spearman-rank correlations adjusted for multiple comparisons. Solid lines indicate significant relationships ($p<0.05$) and dashed lines are marginal ($p<0.1$).
Figure 3.7 Principal components analyses for the main traits investigated here from (A) the controlled environment experiment on 12 cultivars of soybean and (B) the field experiment on eight of the same cultivars. Light saturated assimilation ($A_n$), stomatal
conductance to CO\textsubscript{2} \((g_{s-co2})\), mesophyll conductance \((g_m)\), total electron transport \((J_{cal})\), leaf mass per area \((LMA)\), and leaf dry matter content \((LDMC)\) were used in the fitting for A and B. Additionally in B, nitrogen content per leaf area \((N_{area})\) and carbon-13 to carbon-12 isotope ratio \((\delta^{13}\text{C})\) were used in the fitting. Intrinsic water use efficiency \((A_v/g_s; \text{ dashed line})\) was mapped in the trait space in both A and B. In A different symbols indicate different cultivars, while in B they indicate different soybean growth stages.
Chapter 4. Physiological Changes Across a Selection of Historical Soybean Cultivars

Abstract

Soybean yields in the United States have steadily increased over the past century of breeding for yield. Selection to increase yields was contingent upon improving a host of associated agronomic and physiological traits. Several studies have documented an increase in soybean photosynthetic rate driven by greater stomatal conductance among more modern cultivars when compared to older cultivars. Here we set out to test if the previously observed historical increases in leaf-level photosynthesis and stomatal conductance scale up to the soybean canopy. If modern soybean cultivars conduct more water vapor, then increasing canopy conductance would be associated with greater transpirational water loss. Greater canopy transpiration would lower canopy temperature and heat stress, but increase crop water use. Thirteen cultivars spanning 75 years of soybean improvement were grown in 52, two square meter field plots. We measured leaf and canopy conductance and temperatures with thermal imaging, assessed leaf stress with chlorophyll fluorescence, and soil and plant water status as predawn and midday leaf water potentials. We further measured leaf-level gas exchange twice during the season to allow for comparison with a prior study on historical changes in yield with photosynthetic efficiency. We found that more modern cultivars were less water stressed at midday, yet found no evidence these modern cultivars lowered soil moisture to a greater extent. Modern cultivars had greater canopy conductance, and lower canopy temperatures than older cultivars. As a result, older cultivars displayed more pronounced heat stress. No trends were detected across the historical cultivar set for mesophyll conductance, nor maximum carboxylation or maximum electron transport capacities,
indicating improvements in photosynthetic capacity are relatively limited. Leaf photochemical efficiency and linear electron transport were improved in modern cultivars, suggesting that the increase in canopy conductance directly increases photosynthesis through a reduction in CO$_2$ diffusional limitation and indirectly by reducing leaf temperature and heat stress. Continued selection to increase canopy conductance for yield enhancement will inevitably lead to greater crop water use. However, there is little evidence for broad gains in photosynthetic efficiency in soybean indicating ample capacity for further improvement in yield by increasing photosynthetic efficiency.

**Introduction**

Since American farmers began growing soybean (*Glycine max*) in earnest during the early 20$^{th}$ century, yields have increased from a nationwide average of 868 kg ha$^{-1}$ in the first decade for which data is available (beginning in 1924) to 2914 kg ha$^{-1}$ for the most recent decade (ending in 2015): a linear increase of 23.9 kg ha$^{-1}$ yr$^{-1}$ (USDA, NASS; Figure 4.S1). These on-farm yield increases result from improved soybean genetics (i.e., new cultivars), improved agronomic practices (e.g., better weed control and earlier planting), the interaction of genetics and agronomy (Cober et al., 2005; Rowntree et al., 2014), and elevated atmospheric CO$_2$ concentrations (McGrath and Lobell, 2011; Sakurai et al., 2014). Estimates of the proportion of on-farm yield increase due to these various factors indicate that at least two-thirds of the increase results from genetic improvements in the cultivars used by farmers (Rincker et al., 2014; Specht et al., 2014). We can understand how breeding has changed yields by determining if agronomic traits among cultivars released over time are different in common environments. With this approach differences in trait values are assessed with respect to the year of release
(YOR) of the individual cultivars. Several such studies in soybean offer insight into the traits selected coincident with yield. The proximate trait responsible for greater yield is a greater number of seeds per plant in modern cultivars (Morrison et al., 2000; Jin et al., 2010; Rincker et al., 2014), likely resulting from lower seed and pod abortion. Several agronomic and physiological traits are ultimately responsible for the increase in seeds per plant and crop yield potential.

All determinants of yield potential have increased in response to soybean breeding. Yield potential is the effective seed yield of a cultivar when grown under ideal conditions without biotic or abiotic limitations or stresses (Evans and Fischer, 1999) and can be decomposed into several components (Monteith, 1977) that are more easily associated with agronomic and physiological traits. In this framework, yield potential is a function of the total energy in solar radiation at the canopy ($S$), the portion of that radiation intercepted by the canopy ($\varepsilon_i$), the efficiency of converting $\varepsilon_i$ to biomass ($\varepsilon_c$), and the efficiency of partitioning $\varepsilon_c$ to seed ($\varepsilon_p$; commonly, harvest index) (Monteith, 1977; Zhu et al., 2010). Modern cultivars mature 8-10 days later than cultivars from the 1920’s (Rincker et al., 2014; Rowntree et al., 2014; Koester et al., 2014), and thus have longer growing seasons allowing greater season-long $S_i$. A consistent decrease in lodging is observed with YOR (Morrison et al., 2000; Jin et al., 2010; Rincker et al., 2014; Koester et al., 2014; Keep et al., 2016). Lodging occurs when stems bend under the weight of the plant, which lowers intercepted solar radiation. Less lodging improves yield by increasing $\varepsilon_i$ (Koester et al., 2014). A modest positive relationship is observed between leaf photosynthetic rate and cultivar YOR (Morrison et al., 1999; Jin et al., 2010; Cui et al., 2016; Koester et al., 2016; Li et al., 2017), contributing to a greater $\varepsilon_c$ in modern soybean lines (Koester et al., 2014). Finally, harvest index has improved drastically with
modern cultivars partitioning upwards of 50% of aboveground biomass to seed (Morrison et al., 1999; Jin et al., 2010; Zhu et al., 2010; Koester et al., 2014). A cogent argument can be made that in current soybean cultivars $\varepsilon_i$ and $\varepsilon_p$ are near their theoretical upper limits and future gains in yield potential will require selection to further increase $\varepsilon_c$ (Zhu et al., 2010; Ainsworth et al., 2012).

The balance of photosynthesis (carbon uptake) and respiration (carbon loss) determines the $\varepsilon_c$ component of yield potential. Several observations of genetic gain in net photosynthetic rate with YOR are reported in the literature (Morrison et al., 1999; Jin et al., 2010; Liu et al., 2012; Cui et al., 2016; Koester et al., 2016; Li et al., 2017), and are associated with a concomitant increase in stomatal conductance (Morrison et al., 1999; Liu et al., 2012; Koester et al., 2016; Li et al., 2017). Stomatal conductance determines the rate of CO$_2$ diffusion into, and water diffusion out of, the leaf. Greater stomatal conductance is acknowledged as a component of increasing yields in many C$_3$ species (Roche, 2015) because it allows continued CO$_2$ diffusion into leaves during the warmest portion of the day around solar noon when photosynthetically active radiation is greatest. Stomatal conductance is also directly related to transpiration rates and latent heat loss from canopies: greater stomatal conductance results in greater heat loss, facilitating the cooler canopies observed in modern soybean cultivars (Keep et al., 2016). Cooler canopies in turn may benefit the photosynthetic reactions by reducing heat stress, keeping quantum efficiencies high, and maintaining positive carbon balance longer during the warmest part of the afternoon. On-farm yields of soybean are sensitive to water availability as highlighted by the yield deviation between irrigated and rainfed systems (Specht et al., 1999, 2014). If increased leaf-level stomatal conductance scales to increase canopy conductance of water vapor, modern cultivars may exhaust soil
water more rapidly under dry conditions and explain the decrease in yield stability among environments with soybean YOR (Rincker et al., 2014).

Looking forward to improve yields in the future requires understanding which traits have simultaneously changed with increased yield, \( \varepsilon_c \), and photosynthetic rate historically. Here we tested several hypotheses in a selection of 13 U.S., maturity group III (MGIII), soybean cultivars released between 1924 and 1998 (Table 4.1). We hypothesized that (i) the positive relationship between leaf-level stomatal conductance and YOR scales up to the canopy, (ii) increased canopy conductance results in a cooler canopy reducing leaf-level heat stress, (iii) lower heat stress results in greater quantum efficiency of photosystem II and linear electron transport, and (iv) greater stomatal conductance is facilitated by an increase in leaf water potential resulting from greater exhaustion of soil water by modern cultivars. Canopy temperature and conductance were measured and estimated with infrared thermography on field plots. We assessed cultivars for heat-related stress with chlorophyll a fluorescence induction kinetics (Stirbet and Govindjee, 2011). We compared canopy and leaf responses by measuring leaf level photosynthesis, stomatal conductance, and photosynthetic efficiency twice during the season. Soil moisture was estimated throughout the growing season. Plant water status was determined by measuring predawn and midday leaf water potentials during times of both relatively high and low soil water availability.

**Methods**

Seeds of thirteen cultivars of soybean (\textit{Glycine max}) spanning 75 years of public breeding were obtained from the United States Department of Agriculture Germplasm Resource Information Network (Table 4.1). To increase seed supplies, all cultivars were grown and harvested in the field during the 2015 growing season. The field site is
located in the Hocking River valley of Southeast Ohio where the dominant soil series is a Fluventic Eutrudept silt loam (Hay1AO; USDA NRCS). According to a micro-meteorological tower at the field site, for 2016, total growing season precipitation was 251 mm, the mean daily temperature was 22.2 °C, and the mean daily minimum and maximum temperatures were 16.5 and 28.6 °C (Figure 4.1). On May 16-18, 2016 four 1x2 meter randomly arranged plots of each cultivar were planted resulting in 52 total plots. Each plot consisted of five, 1 m long rows (0.45 m row spacing), and plots were separated from each other by 0.6 m. Seeds were planted to a depth of 3 cm at a rate twice the desired density to allow for poor germination (Koester et al., 2014) of some cultivars. After germination each row within a plot was thinned to 12 plants, providing 7.6 cm spacing between plants within rows and yielding a total plant population within plots of 60. A final thinning was performed when plants were at approximately V1, it was noted that root systems were already extensively nodulating (personal observation). The field was continuously hand-weeded throughout the season and was completely rainfed.

Soil moisture of each plot was measured throughout the growing season. Two measurements of soil water content to 10 cm depth were averaged for each plot – between the second and third rows and between the third and fourth rows – with a soil moisture probe (HS2 with CS659 probes, Campbell Scientific, Logan, UT, USA). Soil water content was measured on 10 days of the year (DOY): 165, 172, 175, 179, 195, 200, 207, 213, 221, and 243. Predawn leaf water potentials were measured with a pressure chamber (Model 1000 Pressure Chamber, PMS Instrument Co., Albany, OR, USA) on one plant from each plot as a proxy of plant-available soil moisture content. The predawn measurements were made on four dates (DOY: 217, 222, 241, 244) late in the season when weather conditions were warm and dry (Figure 4.1).
217, 222, 244) leaf water potentials were also measured at midday to assess canopy water stress.

Leaf-level gas exchange was measured under tightly controlled conditions when plants were transitioning from vegetative to reproductive growth (V6-R1) and again during mid-to-late reproductive growth (R4-R5). Young, fully expanded, upper canopy leaves were sampled from each plot before sunrise, and were kept in darkness during transport to the laboratory with their petioles submerged in water. Once in the laboratory, petioles were re-cut underwater and the leaves were stored in the dark until ready for measurement. Twenty minutes prior to measurement leaves were placed under an LED panel (300W Diamond Series, Advanced LED Illuminatum Inc., Hiwasse, AR, USA) at a photosynthetically active radiation (PAR) of approx. 1500 µmol photons m$^{-2}$ s$^{-1}$. CO$_2$-response curves were then measured with a photosynthesis system (LI-6400XT, LiCor Inc., Lincoln, NE, USA) fitted with the 6400-40 fluorescence head. Leaves were stabilized at an ambient [CO$_2$] of 400 µmol mol$^{-1}$, PAR of 1800 µmol photons m$^{-2}$ s$^{-1}$ (10% blue), flow rate of 300 µmol s$^{-1}$, vapor pressure deficit of <1.3 kPa, and leaf temperature of 25°C maintained by adjusting the 6400 block temperature. After at least 30 min. and once leaves had stabilized a point was logged and the ambient [CO$_2$] was iteratively run through the sequence 400, 325, 250, 175, 100, 50, 400, 400, 500, 650, 950, 1250, 1600, and 2000 µmol mol$^{-1}$. At each CO$_2$ setpoint all gas exchange parameters were logged and a chlorophyll fluorescence routine was initiated and logged using the multiphase flash protocol (Loriaux et al., 2013) to obtain steady-state ($F_s$) and maximum ($F_m$) chlorophyll fluorescence. After the CO$_2$-response curve was complete, chamber conditions were returned to ambient and the leaf was allowed to re-acclimate. Once stomatal conductance ($g_{st}$) and net assimilation ($A_n$) returned to their steady-state
levels the air stream to the 6400 was switched from room air to a humidified tank of N\textsubscript{2} with 1\% O\textsubscript{2}. A second CO\textsubscript{2}-response curve was then initiated with only sub-ambient CO\textsubscript{2} concentrations: 400, 325, 250, 175, 100, and 50 µmol mol\textsuperscript{-1}. Diffusional leaks of CO\textsubscript{2} into and out of the measurement chamber were corrected with the method of Flexas et al. (2007). Briefly, identical CO\textsubscript{2}-response curves were measured on heat-inactivated leaves and their apparent photosynthetic rate was subtracted from the photosynthetic rates of experimental leaves. The intercellular CO\textsubscript{2} concentration of all observations was then recalculated given the new A\textsubscript{n} according to the equations used in the 6400 (Using the LI-6400 / LI-6400XT Portable Photosynthesis System, Version 6, 2012).

Immediately following gas exchange leaf absorptance (\(\alpha\)) was measured at the wavelengths (± 5 nm) corresponding to the LED peaks of the 6400 light source (470 and 665 nm, adjusted to account for only 10\% of the PAR at 470 nm) with leaf-clamp integrating spheres and a spectroradiometer (Jaz Spectroclip, Ocean Optics, Inc., Dundee, FL, USA). Absorptance was measured at three locations across the leaves, avoiding major veins, and averaged. The spectroradiometer was out for service during the R4-5 measurement campaign, so leaf \(\alpha\) was estimated by its relationship with relative chlorophyll content, measured with a chlorophyll meter (SPAD-502, Spectrum Technologies, Inc., Aurora, IL, USA). Since the SPAD values from the V6-R1 stage were lower than those from R4-5, additional data from a diverse set of soybean cultivars (Tomeo and Rosenthal, 2017) was used to regress known \(\alpha\) with SPAD, and \(\alpha\) was estimated from this relationship as: \(\alpha = 0.0032736 + \text{SPAD} \times 0.7677809 \ (R^2=0.56, \ p<0.001)\). Seven, 1 cm diameter leaf punches were then taken, again avoiding major veins, massed for fresh weight, dried at 60°C for >72 h, and massed for dry weight. Leaf mass per area (LMA) was calculated as the ratio of dry-weight to area.
Several leaf physiological parameters were then estimated from the CO$_2$ response curves. First the respiration rate in the light ($R_d$) was estimated from the CO$_2$ response curves by fitting to the C$_3$ biochemical model of photosynthesis (Farquhar et al., 1980) with the plantecophys R package (Duursma, 2015). The quantum yields of photosystem II ($\Phi_{\text{PSII}}$) and CO$_2$-fixation ($\Phi_{\text{CO2}}$) were calculated as:

$$\Phi_{\text{PSII}} = (F_m - F_s)/F'_m$$  
Equation 4.1

$$\Phi_{\text{CO2}} = (A_N + R_d)/(a\text{PAR})$$  
Equation 4.2

Parameter estimates from a linear regression of $\Phi_{\text{PSII}}$ with $\Phi_{\text{CO2}}$ using the 1% O$_2$ curve were used to calibrate $\Phi_{\text{PSII}}$ to account for any alternative electron sinks, which are assumed to be minimal under non-photorespiratory conditions:

With 1% O$_2$ curve:  
$$\Phi_{\text{PSII}} = k \Phi_{\text{CO2}} + b$$  
Equation 4.3

With 21% O$_2$ curve:  
$$\Phi_{\text{cal}} = 4*(\Phi_{\text{PSII}} - b)/k$$  
Equation 4.4

The total linear electron transport rate was then calculated using the $\Phi_{\text{cal}}$:

$$J_T = \Phi_{\text{cal}}\times\text{PAR}$$  
Equation 4.5

and  
$$J_T = J_C + J_O$$  
Equation 4.6

where $J_C$ and $J_O$ are partitions of $J_T$ destined to support carboxylation and oxygenation reactions respectively. With the assumption that four electrons are required for each carboxylation and eight for each oxygenation, $J_C$ and $J_O$ are:

$$J_C = 4*(A_N + R_d + PR)$$  
Equation 4.7

$$J_O = 8\times PR$$  
Equation 4.8

where $PR$ is the rate of CO$_2$ release resulting from the photorespiratory pathway and is calculated as:

$$PR = [(J_T - 4*(A_N + R_d))/12]$$  
Equation 4.9

Combining equations allowed us to estimate $J_C$:
The mesophyll conductance to CO\textsubscript{2} ($g_m$) was then estimated with the variable-$J$ method (Harley et al., 1992):

\[ g_m = A_N / \left\{ C_i - \left[ \Gamma^*(J_T + 8(A_N + R_d)) / [J_T - 4(A_N + R_d)] \right] \right\} \]

where $\Gamma^*$ represents the photorespiratory CO\textsubscript{2} compensation point. Here we used the mean apparent $\Gamma^*$, 4.082 Pa, from Tomeo and Rosenthal (2017). An estimate of $g_m$ was obtained for each $A_N$-$C_i$ pair. We chose to use only the estimate calculated from points measured at an ambient [CO\textsubscript{2}] of 325 µmol mol\textsuperscript{-1} following the logic of a recent study in soybean (Tomeo and Rosenthal, 2017). With these estimates of $g_m$, $A_N$-$C_i$ curves were converted to $A_N$-$C_c$ curves:

\[ C_c = C_i - A_N/g_m \]

The $A_N$-$C_c$ curves were then fit to the C\textsubscript{3} model of photosynthesis, again using plantecophys (Duursma, 2015), to estimate the maximum carboxylation capacity ($V_{C_{\text{max}}}$) and maximum electron transport capacity ($J_{\text{max}}$).

Fast chlorophyll \textit{a} fluorescence induction kinetics were measured to estimate heat stress on leaves in each plot. Fluorescence was measured in the afternoon on three days of increasing daily maximum temperature (DOY 235, 238, and 239) with the final day corresponding to the date with the highest daily maximum temperature over the whole growing season (Figure 4.1). Fully expanded, upper canopy leaves from each plot were fitted with dark-adaptation clips and allowed to acclimate for ≥20 minutes. Leaves were then illuminated continuously with a 3500 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} measurement light and chlorophyll \textit{a} fluorescence was continuously monitored for 10 s using a handheld fluorimeter (Pocket PEA, Hansatech Instruments Ltd., King’s Lynn, Norfolk, U.K.). The area above the fluorescence curve from the moment the light was switched on ($F_o$) until
maximum fluorescence \((F_m)\) was achieved (OJIP\textsubscript{Area}), the fluorescence signal 300 \(\mu\)s after the light was switched on (K-step), and the ratio of variable to maximum fluorescence \((F_v/F_m = [F_m - F_o]/F_m)\) were extracted from continuous traces of chlorophyll a fluorescence (Figure 4.S2). \(F_v/F_m\) is a commonly used metric of general leaf stress (Maxwell and Johnson, 2000), OJIP\textsubscript{Area} is sensitive metric of the pool size of the quinine-A electron acceptor on the reducing end of PSII, and the K-step is a specific indicator of heat stress (Brestic and Zivcak, 2013).

Peak canopy leaf area index (LAI), i.e., leaf area per unit ground area, was measured indirectly with an optical canopy analyzer (LAI-2200, LiCor Inc., Lincoln, NE, USA). Measurements were taken on each individual plot immediately before sunset on DOY 214 and 215. Following the manufacturer’s recommendations, prevailing light conditions of the sky were measured before and after logging five points along an angled transect across the middle three rows of each plot below the canopy. The instrument’s optical sensor was fit with the 90° cap and two of the five measurement angles were not used in the analysis such that the estimated LAI was indicative of the plot being measured and not the adjacent plots.

Plot canopy temperatures were measured with infrared thermography. Images of each plot were taken from approx. 1.5 m above the canopy with a dual infrared and visible digital camera (E6, FLIR Systems Inc., Wilsonville, OR, USA). In addition to the canopy itself, each image also contained wet \((T_{\text{wet}})\) and dry reference \((T_{\text{dry}})\) ‘leaves,’ constructed to mimic leaves with infinite and no transpiration, respectively. The dry reference leaf consisted of an actual soybean leaf coated in petroleum jelly so that no water would be lost from the leaf. This dry reference was supported on an expanded polystyrene frame that supported the leaf as a flat plane, but also allowed maximum
airflow around both surfaces of the leaf and kept the leaf suspended so that it could
loose infrared radiation from both surfaces. The wet reference was made from a
15x15x5 cm expanded polystyrene block wrapped in three layers of white non-viscose
polyester fabric and kept floating in water with its upper surface exposed to wind. The
expanded polystyrene both kept the fabric floating and thermally insulated the upper
surface from the water it was floating in. Wet and dry references were mounted on a
tripod and moved up and down as necessary to keep them just above canopy height.
Plot-identification cards were also mounted on the tripod for latter identification of
images (Figure 4.S3). Images were taken in the afternoon on two dates, DOY 220-221
and 235-236. The plots themselves were arranged randomly such that thermal images
were taken in a random fashion with respect to cultivar identity, and different sampling
strategies were used on the two dates to avoid confounding time of day with
measurements of interest. Ambient air temperature ($T_{air}$; HMP60, Viasala, Vantaa,
Finland) and wind speed ($\mu$; 1733 anemometer, Adafruit Industries, NY, NY, USA) were
continuously monitored and logged at 15 s intervals (LI-1400, LiCor Inc., Lincoln, NE,
USA).

Thermal images were analyzed with FLIR Tools software (v5.716168.1001). The
temperatures of the wet ($T_{wet}$) and dry ($T_{dry}$) references, and four obviously sunlit leaves
were extracted. Temperatures for the four leaves were averaged to give an estimate of
sunlit canopy temperature ($T_{can}$). With the canopy and reference temperatures we also
estimated canopy-scale stomatal conductance ($g_{s-canopy}$) (Leinonen et al., 2006; Guilioni
et al., 2008; Jones, 2013). The stomatal resistance to water vapor ($r_s$, mm s$^{-1}$) was
calculated here as in Case 7, of Table 1 in Guilioni et al., (2008):

$$r_s = [r_{aw} + (s'/\gamma)*r_{HR}]*(T_{can} - T_{wet})/(T_{dry} - T_{can})$$

Equation 4.13
where $s$ is the slope of the saturation vapor pressure curve and dependent on temperature with Tetens equation (Murray, 1967), $\gamma$ is the psychrometric constant as a function of air temperature (Appendix 3. Jones, 2013). Boundary layer resistance to water vapor($r_{aw}$), and parallel resistance to heat ($r_{ah}$) and energy ($r_{R}$) transfer ($r_{HR}$) were calculated as:

$$r_{HR} = \frac{(r_{ah} * r_{R})}{(r_{ah} + r_{R})} \quad \text{Equation 4.14}$$

$$r_{aw} = 0.92 * r_{ah} \quad \text{Equation 4.15}$$

$$r_{ah} = \alpha_1 * (d/\mu)^{0.5} \quad \text{Equation 4.16}$$

$$r_{R} = (\rho * c_p)/(8 * \varepsilon_L * \sigma * T_{air}^3) \quad \text{Equation 4.17}$$

where the constant 0.92 accounts for the inseparability of stomatal and boundary layer resistances (Guilioni et al., 2008), $\alpha_1$ is a unit correction (for the case here with hypostomatous leaves a value of 200 s m\(^{-1}\) was used), $d$ is leaf characteristic dimension with 0.072 m used here as an average leaf width, $\mu$ is wind speed, $\rho$ is air density, $c_p$ is specific heat capacity of air, $\varepsilon_L$ is leaf emissivity, and $\sigma$ is the Stephan-Boltzmann constant. The entire function $r_R$ was simplified as a linear function of temperature over the temperature range encountered during image acquisition ($r_R = -2.46 * T_{air} + 261$; Jones, 2013). Stomatal resistance was then converted to $g_{s-canopy}$ with the appropriate units by assuming the conversion factor was linearly related to temperature over the range in $T_{air}$ encountered ($g_{s-canopy} = 1/r_s * 43.58 - T_{air} [\text{mmol m}^{-2} \text{s}^{-1}]$).

Data Analysis – Means of all photosynthetic gas exchange traits, leaf water potentials (predawn, midday, and their differential), $T_{can}$, $g_{s-canopy}$, LAI, OJIP\text{Area}, and K-step were calculated for each cultivar (i.e., YOR). Least squares regressions were then performed comparing the mean of each trait against YOR and the growth stage or date of measurements as fixed effects. Soil volumetric water content was compared among
cultivars with a repeated measures ANOVA using day-of-the-year (DOY) as the time stamp. All data processing and analyses were performed in the R statistical environment v3.3.2 (R Core Team, 2015).

**Results**

Photosynthetic CO$_2$-response curves were measured on excised leaves in the laboratory to assess leaf-level physiological performance and photosynthetic parameters under tightly controlled conditions. The quantum yield of photosystem-II ($\Phi_{PSII}$; $p<0.05$), calibrated total electron transport rate ($J_T$; 0.486 µmol e$^{-}$ m$^{-2}$ s$^{-1}$ year$^{-1}$, $p<0.05$), photorespiration ($PR$; 0.036 µmol CO$_2$ m$^{-2}$ s$^{-1}$ year$^{-1}$, $p<0.01$), the CO$_2$ concentration gradient between the substomatal cavity and the chloroplast stroma ($C_i$ - $C_c$; 0.178 µmol CO$_2$ mol$^{-1}$ air year$^{-1}$, $p<0.05$), and intrinsic water use efficiency ($A_{N}/g_{sH}$; 0.122 µmol CO$_2$ mol$^{-1}$ H$_2$O year$^{-1}$, $p<0.05$) were each positively correlated with YOR (Figure 4.3). Carbon dioxide concentrations in substomatal cavities ($C_i$; -0.133 µmol CO$_2$ mol$^{-1}$ air year$^{-1}$, $p<0.05$) and in the chloroplast stroma ($C_c$; -0.311 µmol CO$_2$ mol$^{-1}$ air year$^{-1}$, $p<0.01$) were both negatively correlated with YOR (not shown). Notably, no relationships were detected between YOR and the enzymatic driver of photosynthesis, the maximum carboxylation capacity of Rubisco ($V_{Cmax}$) or the maximum potential electron transport rate ($J_{max}$), which drives the regeneration of the RuBP (Figure 4.S4), or steady-state photosynthesis ($A_{N}$) itself. Likewise, there was no relationship between YOR and stomatal or mesophyll conductance (Figure 4.S5) measured in the laboratory despite significant relationships between YOR and $C_i$, $C_c$, and $C_i$ - $C_c$.

Plot-level soil volumetric water content (vol/vol; VWC) to a depth of 10 cm was measured throughout the growing season to establish if cultivars differentially reduced soil water. Soil VWC varied between 3.65 and 28.7%, and differed substantially across
the growing season ($p<0.001$), declining to an average of 5.73% at day of the year 243, and after which the soil surface was too dry and hard to safely insert the probes. The average plot VWC over the growing season was 13.9% (Figure 4.4) and no differences were detected among cultivars or across cultivar YOR dates. Predawn leaf water potential ($\Psi_{pd}$), an indicator of soil water content available in the rooting zone, was measured on four dates: two when soil-VWC was relatively high, and two when soil-VWC was low (Figure 4.4). There was a non-significant trend toward higher (less negative) $\Psi_{pd}$ in more modern cultivars ($0.337$ kPa year$^{-1}$, $p=0.06$; Figure 4.5).6). There was a non-significant trend toward higher (less negative) $\Psi_{pd}$ in more modern cultivars ($0.337$ kPa year$^{-1}$, $p=0.06$; Figure 4.5).

Canopy water stress was determined as midday leaf water potentials ($\Psi_{md}$), which were measured on three dates. Modern cultivars had higher $\Psi_{md}$ with the predicted $\Psi_{md}$ for the most recent cultivar $0.075$ MPa higher than the oldest cultivar ($0.99$ kPa year$^{-1}$, $p<0.001$; Figure 4.5). Predawn water potentials were measured the morning of each $\Psi_{md}$ assessment. The differential between $\Psi_{md}$ and $\Psi_{pd}$ declined with YOR by nearly 1% per year ($p<0.001$)(Figure 4.5).

Peak season leaf area index (LAI) averaged $7.2$ m$^2$ m$^{-2}$. LAI did not differ among cultivars ($p>0.1$) nor was there any trend with YOR ($p>0.1$)(Figure 4.5).

Canopy temperatures ($T_{can}$) and stomatal conductance ($g_{s-canopy}$) were measured at the plot-scale with infrared thermography. Relative to air temperature, modern cultivars had canopies significantly cooler than older cultivars ($-0.19$ °C decade$^{-1}$, $p<0.001$; Figure 4.5). Lower temperatures were likely a result of the greater $g_{s-canopy}$ in modern cultivars ($0.021$ mol H$_2$O m$^{-2}$ s$^{-1}$ decade$^{-1}$, $p<0.01$; Figure 4.5). Fast chlorophyll $a$ fluorescence induction kinetics was used to test for leaf-level stress in the canopy. Modern cultivars had more area above their fluorescence induction curves ($p<0.001$; Figure 4.5). The quantity of fluorescence $300$ µs after the measuring light hits the leaf, referred to as the
K-step, is specifically indicative of heat stress, with greater fluorescence indicating greater stress. Older cultivars had a higher K-step (p<0.05; Figure 4.7).

**Discussion**

We found support for the first three of our hypotheses and partial support of the fourth. Confirming the results of Keep et al., (2016), we observed lower canopy temperatures in modern cultivars. Further, this is the first study attributing a reduction in canopy temperature to greater canopy conductance in modern soybean cultivars. We saw a decline in general leaf stress as determined by initial fluorescence response (i.e., OJIP Area), and specifically heat stress (K-step) in modern compared to older cultivars during warm afternoons. The quantum efficiency of PSII, which measures the proportion of photons utilized, and linear electron transport rate, which generates reducing power for the Calvin cycle, both appear to have increased with historical yield gains in soybean. Modern cultivars apparently use more water per unit area as they have greater $g_{s}$-canopy and similar leaf area. Higher canopy water use by modern cultivars was, however, not associated with lower soil moisture as estimated directly with measurements of soil moisture (vol/vol) or with plant water status determined with predawn leaf water potentials.

Increasing stomatal conductance simultaneous with genetic-yield gain in soybean is now well established (Morrison et al., 1999; Liu et al., 2012; Koester et al., 2016; Li et al., 2017). More generally, stomatal conductance is commonly co-selected with yield in C$_3$ crops (Roche, 2015). To our knowledge ours is the first study to show that the increase in leaf-level $g_{s}$ with soybean cultivar release date extends to the canopy ($g_{s}$-canopy; Figure 4.6). Since soybean yields are dependent on water availability (Specht et al., 1999, 2014), selection for yield contingent on greater $g_{s}$-canopy, if continued comes
with the tradeoff of greater crop water use. With increasing \( g_{\text{c-canopy}} \) the volume of water transpired by the canopy, and the extent that soil water is depleted, both increase. Leaf-level \( g_s \) and \( g_{\text{c-canopy}} \) are both measured on a leaf area basis, so the total leaf area of a canopy will modulate the quantity of water it transpires. While we detected no relationship between peak season leaf area index (LAI) and YOR (Figure 4.S7), studies with short-season Canadian (Morrison et al., 1999) and Chinese (Jin et al., 2010) soybean did observe a decline in LAI with YOR. Assuming our \( g_{\text{c-canopy}} \) estimates only apply to the single uppermost layer of leaves, we can estimate that modern cultivars were conducting an additional 0.157 mol H\(_2\)O m\(^{-2}\) s\(^{-1}\). Even though the modern cultivars investigated here did use more water, there was no indication they differentially reduced soil water content (Figure 4.4). Additionally, predawn leaf water potentials, which can be used as a conservative estimate of soil water potential for similar plants (Donovan et al., 2003; Rosenthal et al., 2005), were not detectably different by YOR indicating that modern cultivars had at least equivalent access to soil water as old cultivars (Figure 4.S6). There is evidence that selection for yield in Chinese soybean germplasm has resulted in more active root systems capable of transporting a greater volume of water to the canopy (Cui et al., 2016). In a particularly revealing study where the shoots of older soybean cultivars were grafted onto newer ‘elite’ rootstocks, Li et al., (2017) demonstrate that \( g_s \) and net photosynthesis of older cultivars was higher on newer rootstocks. It may be that greater root activity (Li et al., 2017) and greater stomatal conductance (this study and others), which both improve plant water status, were co-selected to increase yield during US soybean improvement, but this requires further investigation.

We show that historical increases in \( g_{\text{c-canopy}} \) are associated with decreases in canopy temperature, which is consistent with recently published work on many cultivars (Keep et
al., 2016) including those in this study. Keep et al., (2016) saw a decline in canopy temperatures with soybean cultivar YOR over two seasons and under irrigated and rainfed treatments, suggesting our gs-canopy results are not isolated to the single environment sampled or just the cultivars in our study. Canopies of the most recently released cultivars averaged 1.4°C cooler than the oldest cultivars (Figure 4.6), which is consistent with the 2°C cooler canopies observed in cultivars released over a 90 year period by Keep et al. (2016). Though a fairly small temperature differential, we hypothesize it helped to protect more recent cultivar releases from heat stress that affected the older cultivars (Figure 4.7). We also found an increase in midday leaf water potential with YOR (Figure 4.5). Even among cultivars available 50 years ago there was evidence that recent introductions had higher midday leaf water potentials (Boyer et al., 1980), demonstrating a reduction in leaf water stress early in soybean improvement. Leaves with less water stress can maintain greater gs and transpiration longer as found here (Figure 4.6) and elsewhere (Morrison et al., 1999; Liu et al., 2012; Koester et al., 2016; Li et al., 2017).

More modern cultivars also exhibit a greater capacity for coping with excess excitation energy. A trend of increasing leaf chlorophyll concentration with soybean YOR was observed in the same cultivars used in this experiment (Koester et al., 2016), and in a larger selection spanning both MGIII and MGIV (Keep et al., 2016). Leaves with more chlorophyll will inevitably absorb more photosynthetically active radiation. Mechanisms must exist for utilizing the extra excitation energy absorbed by the photosystems. Li et al., (2017) measured the quantum yield of photosystem-II ($\Phi_{\text{PSII}}$) on dark-adapted leaves and demonstrated an increase of $\Phi_{\text{PSII}}$ with YOR. We confirm this result with gas exchange calibrated $\Phi_{\text{PSII}}$ estimates, finding that more recently released cultivars had a
greater $\Phi_{\text{PSII}}$ and total electron transport rate ($J_T$) (Figure 4.2). Therefore, modern cultivars are effectively using a greater share of absorbed photons to generate the reducing power that drives the light-independent reactions. Alternatively, a lower $\Phi_{\text{PSII}}$ and $J_T$ in older cultivars may indicate more photodamage to PSII arising from their lower ability to effectively vent excess excitation energy. Rising $g_s$ (Figure 4.6; Morrison et al., 1999; Liu et al., 2012; Koester et al., 2016; Li et al., 2017) with YOR indicates a greater capacity to vent excess thermal energy through latent heat loss, which cools canopies (Figure 4.6; Keep et al., 2016) and lowers heat stress (Figure 4.7). Energy venting has also been proposed as one explanation for the persistence of photorespiration despite the substantial energetic losses associated with the pathway (Bloom, 2015). The positive correlation between photorespiration and YOR (Figure 4.2) then offers a second potential avenue for discharging excess energy in modern soybeans. Whether through photochemistry, photorespiration, or latent heat loss, modern cultivars have a superior capacity to use or dissipate excess excitation energy and heat.

Knowing which traits with potential for enhancing photosynthesis have not changed with YOR is as important as knowing which were modified coincident with genetic-yield gain. One such trait is mesophyll conductance. Many have suggested that enhancing leaf mesophyll conductance will result in greater photosynthetic rates (Zhu et al., 2010; von Caemmerer and Evans, 2010; Evans, 2013; Flexas et al., 2013, 2016; Berghuijs et al., 2016; Tomeo and Rosenthal, 2017). We have shown that mesophyll conductance does differ genotypically in food grade soybean cultivars spanning 4 maturity groups (Tomeo and Rosenthal, 2017). Here where we consider cultivars from only one maturity group, we find no evidence for genetic differentiation, nor a trend between mesophyll conductance and YOR (Figure 4.5). Using an alternative and more robust technique to
estimate $g_m$, we confirm the results of Koester et al., (2016). Taken together these findings more strongly support the assertion that $g_m$ has not been systematically improved in a historically representative array of U.S. soybean cultivars. Similarly, there is little indication that maximum carboxylation capacity or maximum electron transport capacity were modified during soybean improvement (Figure 4.S4; Koester et al., 2016). Since net photosynthetic assimilation rates are co-limited by $V_{C\text{max}}$ and $J_{\text{max}}$ (Farquhar et al., 1980; Walker et al., 2014), these traits are obvious targets for further increasing soybean photosynthesis.

**Conclusions**

Leaf and canopy physiology were altered coincident with soybean yield enhancement over the 20th century. We demonstrate that the commonly observed increase in leaf stomatal conductance to water vapor with cultivar year of release scales up to the canopy. Higher canopy conductance in modern soybean cultivars is likely facilitated by root systems with a greater capacity to transport water to shoots (Cui et al., 2016; Li et al., 2017) which reduces leaf water stress, and results in cooler canopies. In turn, cooler canopies exhibit lower heat stress, higher photochemical quantum efficiency of photosystem-II, and higher linear electron transport rates. There is ample room to further improve photosynthesis in soybean (Tomeo and Rosenthal, 2017). Not detecting a relationship between key drivers of photosynthesis (i.e., $g_m$, $V_{C\text{max}}$, $J_{\text{max}}$) and year of release in these genotypes supports the notion that photosynthesis may be further improved in maturity group III soybean genotypes.

**References**

potential in soybean: Potential targets for biotechnological improvement.

Plant, Cell Environ 35: 38–52


Disequilibrium between Predawn Plant and Soil Water Potentials. Ecology 84: 463–470


of Genetic Improvement of Short-Season Soybean Cultivars in Canada.
Agron J 92: 780–784

Years of Genetic Improvement of Short-Season Soybean Cultivars in
Canada. Agron J 91: 685–689

Meteorol 6: 203–204


Maturity Groups II, III, and IV. Crop Sci 54: 1419–1432

Roche D (2015) Stomatal Conductance Is Essential for Higher Yield Potential of

Gradient Across and Active Sand Dune/Desert Boundary in the Great Basin

Rowntree SC, Suhre JJ, Weidenbenner NH, Wilson EW, Davis VM, Naeve SL,
Casteel SN, Diers BW, Esker PD, Conley SP (2014) Physiological and
phenological responses of historical soybean cultivar releases to earlier
planting. Crop Sci 54: 804–816

Sakurai G, Iizumi T, Nishimori M, Yokozawa M (2014) How much has the
increase in atmospheric CO 2 directly affected past. Sci Rep 4: 4078


Table 4.1

Listing of all cultivars used with their respective year of release.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Year of Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dunfield</td>
<td>1923</td>
</tr>
<tr>
<td>Illini</td>
<td>1927</td>
</tr>
<tr>
<td>AK Harrow</td>
<td>1928</td>
</tr>
<tr>
<td>Mandel</td>
<td>1934</td>
</tr>
<tr>
<td>Lincoln</td>
<td>1943</td>
</tr>
<tr>
<td>Adams</td>
<td>1948</td>
</tr>
<tr>
<td>Shelby</td>
<td>1958</td>
</tr>
<tr>
<td>Adelphia</td>
<td>1964</td>
</tr>
<tr>
<td>Williams</td>
<td>1971</td>
</tr>
<tr>
<td>Woodworth</td>
<td>1974</td>
</tr>
<tr>
<td>Zane</td>
<td>1984</td>
</tr>
<tr>
<td>Resnik</td>
<td>1987</td>
</tr>
<tr>
<td>IA3010</td>
<td>1998</td>
</tr>
</tbody>
</table>
Figure 4.1 Temperature and precipitation across the field season. Temperature traces are drawn for daily average (black), daily minimum (light blue), and daily maximum (light red). Black bars indicate daily precipitation and that the dark blue trace is two-week running mean precipitation. The vertical grey shading indicates times when leaves were sampled for gas exchange measurements in the laboratory. The red vertical lines indicate days when thermal images were acquired. The dark green box along the x-axis marks when seeds were planted.
Figure 4.2 The quantum yield of photosystem II (A; $\Phi_{PSII}$), total linear electron transport (B; $J_T$), and photorespiratory CO$_2$ release (C; $PR$) are presented with cultivar year of release. All gas exchange traits were measured twice, once during late-vegetative growth (approximately V6; black symbols) and once during mid reproductive growth (approximately R3; red symbols and fit lines). Lines are least-squares regression fits for individual growth stages: in all cases (A-C) there was a greater intercept during reproductive stage ($p<0.001$).
Figure 4.3 The CO₂ concentration gradient between the intercellular air spaces and chloroplast stroma (A; \( C_i - C_c \)), and intrinsic water use efficiency (B; \( A_n/g_{sh} \)) by year of cultivar release. Black and red symbols represent measurements at late-vegetative and mid-reproductive growth stages respectively. Fit lines are least-squares regression fits: where two lines are presented (B) the intercepts differed between the two growth stages \((p<0.001)\).
Figure 4.4 Volumetric soil water content (VWC) by day of the year (DOY). VWC was measured in each individual plot, from which cultivar means were calculated and plotted here with each cultivar represented by a unique symbol color. The vertical lines indicate the dates that leaf water potential ($\Psi_w$), thermography, and fluorescence were measured.
Figure 4.5 Midday leaf water potentials (A; \( \Psi_{md} \)) and the differential between midday and predawn leaf water potentials (B) by year of cultivar release. Different symbol colors represent day of the year 217 (black), 222 (red), and 244 (green). Lines are least squares regression fits, which differed by DOY (\( p<0.001 \)), but no interactions were detected between DOY and year of release.
Figure 4.6 The differential between canopy and air temperature (A) and canopy conductance to water vapor (B; $g_{s\text{-canopy}}$) by cultivar year of release measured on day of the year 220 (black symbols) and 235 (red). Lines represent least squares regression fits.
Figure 4.7 The integrated area above the chlorophyll $a$ fluorescence induction curve (A; \( \text{OJIP}_\text{Area} \)) and relative fluorescence at 300 $\mu$s after switching on the measuring light (B; \( K\)-step) by cultivar year of release. Symbol colors represent dates of measurements: day of the year 235 (black), 238 (red), and 239 (blue). Lines represent least squares regression fits.
Chapter 5. Conclusions and Future Directions

Summary of preceding chapters

The preceding studies provide an overview of natural genetic variation in photosynthesis among Arabidopsis thaliana ecotypes and soybean cultivars. This knowledge can inform traditional and bioengineering approaches to enhancing C₃ crop photosynthesis, and in soybean this variation demonstrates where future efforts might prove directly useful, and importantly where they will not.

In Chapter 2 we saw that naturally occurring Arabidopsis thaliana ecotypes have divergent photosynthetic physiologies. We measured photorespiration in vivo and found that coordination between the Calvin cycle and photorespiratory pathway scales to the physiological level as a correlation between photorespiratory CO₂ efflux and photosynthetic carbon assimilation. The absence of genetic correlation between photosynthesis and photorespiration suggests that natural selection may work on these traits independently, and artificial selection on one of these traits through traditional breeding strategies may increase photosynthetic efficiency. There were strong genetic correlations between mesophyll conductance and electron transport supporting carboxylation, and between maximum carboxylation ($V_{cmax}$) and electron transport capacities ($J_{max}$), indicating that there is shared inheritance for the primary traits underlying photosynthetic variation among ecotypes. Structural and physiological traits were differentiated by ecotype life-history strategy. Winter annuals generally exhibited greater structural robustness, physiological capacity, and water use efficiency. Integrated water use efficiency was positively correlated with assimilation rates and mesophyll conductance in winter, but not spring, ecotypes. In winter ecotypes a lack of variance for stomatal conductance indicates that if stomatal conductance is held
relatively constant, increasing mesophyll conductance does result in greater water use efficiency.

In Chapter 3 we saw that diverse soybean cultivars from a range of maturity groups have consistently divergent photosynthetic physiologies. The ultimate goal of this work was to demonstrate useful variation that might allow for selection to enhance photosynthesis in the varieties planted by farmers in the United States and Canada. Improving agronomically relevant traits through artificial selections requires genetic variation in the traits of interest, and that the variation is heritable. We demonstrate in soybean that genetic variation exists for mesophyll conductance, and that the variation is highly coordinated with overall leaf photosynthetic physiology and to a lesser extent with coarse metrics of leaf structure. Consistently across environments, genetics contributed to a strong correlation between mesophyll conductance and net photosynthetic rates. Contrary to expectations, phenotypic correlation between stomatal and mesophyll conductance impeded coordination between water use efficiency and mesophyll conductance. Within environments we found that leaves with greater dry mass per area had higher mesophyll conductance and photosynthesis. If this relationship holds for broader collections of soybean cultivars, it suggests a tractable method for selecting genotypes with enhanced photosynthesis. Taken together these results provide evidence that there is genetic variation for mesophyll conductance in soybean that could be selected with traditional breeding techniques, and that doing so would enhance photosynthetic rates.

In Chapter 4 we saw that modern soybean cultivars have greater canopy conductance to water vapor, which then affects leaf temperature and stress. The massive increase in soybean yields over the twentieth century was partially the result of
selection improving many agronomic and physiological traits. Stomatal conductance is one physiological trait selected with yield. We demonstrate that the increase in leaf stomatal conductance to water vapor with cultivar year of release scales up to the canopy. Greater canopy conductance in modern cultivars did not increase leaf water stress and resulted in cooler canopies relative to older cultivars. Cooler canopies exhibited less heat stress, higher photochemical quantum efficiency, and higher linear electron transport rates. Excluding these few photosynthetic traits, we saw little evidence that photosynthesis was comprehensively improved with yield in soybean. For example, no relationships were detected between key drivers of photosynthesis and cultivar release dates, highlighting how little direct enhancement to photosynthesis has occurred during U.S. soybean improvement. The general lack of photosynthetic enhancement in maturity group III soybeans implies that there is still considerable opportunity to do so.

**Consolidated conclusions**

Ultimately we are interested in how to improve photosynthesis in C\textsubscript{3} crops. In Chapter 1 we established that useful traits for breeding programs must meet several criteria. A trait (1) needs to differ with sufficient magnitude among genotypes, (2) must have heritable variance among genotypes, and (3) cannot exhibit the most optimal trait values in widely grown genotypes. With the studies here we can conclude that genetic variation in photosynthetic physiology is plentiful in both ecotypes of the model plant *Arabidopsis thaliana* and cultivars of the valuable crop soybean, and that the variation for many important traits is significantly heritable. In some collections of historical soybean cultivars net photosynthesis has increased, at least marginally, coincident with yield increases (Morrison et al., 1999; Liu et al., 2012; Cui et al., 2016; Li et al., 2017); though in U.S. soybeans, improvements to photosynthesis are quite limited (Chapter 4).
Further, mesophyll conductance was not altered through historical selection for yield in soybean (Chapter 4). Considering that mesophyll conductance has not been improved in U.S. soybean, and that there is significant heritable variation for mesophyll conductance among other soybean cultivars, we can conclude that this trait, and in turn soybean photosynthesis, can still be improved.

In searching for traits to investigate for their potential utility in enhancing photosynthesis, mesophyll conductance was an ideal candidate because of potential to not only decrease diffusional limitations to photosynthesis, but also to improve leaf-level water use efficiency. Unfortunately, we largely found that water use efficiency is unresponsive to genetic changes in mesophyll conductance. Among winter annual type *Arabidopsis thaliana* ecotypes in Chapter 2 stomatal conductance varied minimally while mesophyll conductance varied almost three-fold, permitting a significant positive relationship with integrated water use efficiency, though not intrinsic water use efficiency. No relationship at all was found between water use efficiency and mesophyll conductance among soybean cultivars. When soybeans were grown under field conditions, mesophyll and stomatal conductance decreased at later developmental stages as water availability declined, while water use efficiency increased producing the opposite of the hypothesized effect: water use efficiency tended to decline with increasing mesophyll conductance. If and how water use efficiency was correlated with mesophyll conductance was contingent upon stomatal conductance among both *Arabidopsis thaliana* ecotypes and soybean genotypes. We also failed in both studies to detect a genetic correlation between mesophyll conductance and water use efficiency, indicating that any correlation between the traits was primarily driven by shared environmental responses rather than shared inheritance. There is little potential to
improve water use efficiency through selection on mesophyll conductance in soybean. And, when combined with the results form Arabidopsis thaliana suggests this result may hold for other annual, thin-leaved eudicot species as well.

**Future directions**

Photorespiration represents the largest reduction in photosynthetic efficiency for C$_3$ species (Zhu et al., 2010), and therefore is an obvious target for improvements. A large body of work has described the mechanisms of the photorespiratory pathway, the enzymes and substrates involved, the cell biology of photorespiration, and its molecular interplay with photosynthesis (Peterhansel et al., 2010; Bauwe et al., 2012; Timm et al., 2016). Rubisco specificity for CO$_2$ relative to O$_2$ varies across broad taxonomic ranges (von Caemmerer, 2000), and in some cases within more closely related taxa (Prins et al., 2016). Rubisco specificity also varies in a well known manner with temperature (Bernacchi et al., 2001; Galmés et al., 2014; Prins et al., 2016). Variation in Rubisco specificity has clear implications for the rate of oxygenation reactions and photorespiratory flux. Though, any understanding of genetic variation for photorespiration is lacking in the literature (Nunes-Nesi et al., 2016). In Chapter 2 we showed there is heritable genetic variation for in vivo estimates of photorespiratory CO$_2$ efflux, and that phenotypically photorespiration was correlated with photosynthesis. Future studies should attempt to confirm these findings using alternative methodologies, for example by determining the photorespiratory CO$_2$ compensation point (Brooks and Farquhar, 1985; Walker and Ort, 2015) or the ratio of leaf glycine to serine (Kebeish et al., 2007) in ecotypes shown here to have diverging photorespiratory flux. The lack of a genetic correlation between photorespiration and photosynthesis suggests that selection could disassociate these pathways. If the two pathways can be disassociated,
phenotypic characterization of resultant genotypes would allow us to determine if the associated benefits (sensu Bloom, 2015) proposed for photorespiration actually exist. A model system with natural variation for photorespiration is also a valuable compliment to studies attempting to engineer a bypass of the photorespiratory pathway and its associated costs to leaf carbon balance (Kebeish et al., 2007; Peterhansel and Maurino, 2011; Ort et al., 2015; Betti et al., 2016).

Chapters 3 and 4 indicate that increasing mesophyll conductance in modern soybean cultivars will enhance their photosynthetic rates. There is potential to bioengineer higher mesophyll conductance through over expression of aquaporin proteins in the chloroplast envelope (Hanba et al., 2004; Uehlein et al., 2012). Recently a correlation between mesophyll conductance and carbonic anhydrase activity was observed among natural populations of *Populus trichocarpa* (Momayyezi and Guy, 2017), suggesting a second mechanism for altering mesophyll conductance through targeted overexpression of a single gene. Our data from Chapter 3 show that there is genetic variation for mesophyll conductance in soybean germplasm that could be selected for using traditional breeding strategies. However, the time required to estimate mesophyll conductance with current technology will logistically constrains our ability to select for mesophyll conductance. Even using alternative, more rapid, methodologies than those used in the studies presented here, a maximum of 30 plants can be measured in one day, and then only in a controlled setting (Barbour et al., 2016). We proposed using proxies for mesophyll conductance in Chapter 3, with leaf dry mass per area specifically discussed. Mesophyll conductance was also though highly coordinated with the overall photosynthetic physiology of leaves among both *Arabidopsis thaliana* ecotypes (Chapter 2) and soybean cultivars (Chapter 3), suggesting that other...
physiological traits might provide reasonable proxies. For example, when soybeans were grown in chambers we found significant broad-sense heritability for the correlation between mesophyll conductance and electron transport rate. Among Arabidopsis thaliana ecotypes we found that broad-sense heritability was 30% for the correlation between mesophyll conductance and the quantum efficiency of photosystem-II, a trait perfect for high-throughput phenotyping systems since we can quantify it non-destructively in seconds. The potential to use proxy traits for the estimation of mesophyll conductance should be a primary focus of future work in the area.

References


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Figure 2.S1. Example images of the gas exchange setup. The full LiCor-6400 gas exchange system measuring a soybean plant (A), the ‘ice-cream-cone’ potting strategy allowing leaves to hang over the edge of the pots (B), and a rosette leaf is seen extending into the measurement chamber of the 6400 photosynthesis system (C).
Figure 2.S2. The response of photosynthesis to atmospheric \( \text{CO}_2 \) concentration at ambient (21%; blue symbols and line) and reduced (1%; red symbols and line) atmospheric oxygen concentration. The ratio of oxygenation to carboxylation reactions by Rubisco is strongly influenced by the atmospheric oxygen concentration, such that low (<2%) oxygen effectively inhibits oxygenation events and the resulting photorespiratory flux.
Appendix B: Chapter 3 Supplemental Figures and Tables

Figure 3.S1. Mean (± s.e.) cultivar non-photorespiratory respiration in the light ($R_d$) and apparent photorespiratory CO$_2$ compensation points ($C^*_i$), determined from Laisk curves on $n$=5-8 chamber grown plants.
Figure 3.S2. The reliance of mesophyll conductance ($g_m$) estimates on the value of $\Gamma^*$.

Throughout the manuscript the mean $C_{i^*}$ value was used as a proxy for the true $\Gamma^*$. Here we varied mean $C_{i^*}$ by ±15% and re-estimated $g_m$ for all 12 cultivars used in the controlled environment portion of this study. Mesophyll conductance is strongly dependent on $\Gamma^*$, increasing with greater values of $\Gamma^*$ and decreasing with smaller values. Among cultivar variance in $g_m$ is robust to varying $\Gamma^*$: subtracting 15% from the mean $C_{i^*}$ increases the variance attributable to cultivar by 0.13% (from 38.81%), and adding 15% to the mean $C_{i^*}$ decreases it by 1.26%. In all three cases the inclusion of cultivar-identity significantly ($p<0.05$) increased the variance explained.
Figure 3.S3. Comparisons of $g_m$ calculated using cultivar-specific, and a range of unvarying $R_d$ estimates for (A) the late-vegetative, (B) early-reproductive, and (C) late-reproductive growth stages of field grown soybean cultivars indicated along the x-axis.

When using cultivar-specific $R_d$ values, 11.6% of the variance in $g_m$ is attributable to cultivar, and when using an unvarying $R_d$ (0.5, 1.0, or 1.5) 11.8% of the variance is attributable to cultivar. The small flux of $R_d$ relative to measured $A_N$ in this study results in $g_m$ being nearly unresponsive to $R_d$. 
Figure 3.S4. Mean (± s.e.) absorptance (α) at 470 and 665 nm for each cultivar from the controlled environment experiment (A) and field experiment (B). In A, n=5-6, and in B n=4 at each cultivar-by-growth stage combination except for GS22 where n=3. Note all leaves in A were sampled at the late-vegetative (V4-V5) growth stage. In B a mean (± s.e.) is presented for each growth stage measured on each cultivar: squares are late-vegetative, circles are early-reproductive (R2-R4), and triangles are late-reproductive (R6).
Figure 3.S5. Mean (A) light-saturated assimilation ($A_N$), (B) stomatal conductance ($g_s$), (C) mesophyll conductance ($g_m$), and (D) calibrated electron transport ($J_{cal}$) for cultivars grown in the field. Values are cultivar means ($\pm$ s.e.) for $n=4$ replicates, at the late-vegetative (V4-V5, squares), early-reproductive (R2-R4, circles), and late-reproductive
(R6, triangles) growth stages. Growth stage was a significant fixed effect for \( g_s \) and \( g_m \) \((p<0.05)\), but not for \( A_N \) or \( J_{cal} \) based on linear mixed models with cultivar-identity and row within the field as random effects. Variance due to cultivar was significant \((p<0.05)\) for \( A_N \), \( g_s \), \( g_m \), and \( J_{cal} \) according to likelihood ratio tests.
Figure 3.S6. Daily precipitation, 15-day cumulative precipitation, and mean temperature across the growing season from a meteorological tower located ~200 m from the field experiment. Mean temperature (black line) was derived from hourly-averaged data. Daily precipitation (bars) represents the cumulative precipitation for each 24-h Julian day of the year (DOY). A 15-day moving window of daily-mean precipitation (blue line) is also presented to highlight wet and dry periods. The red line along the x-axis signifies the date of planting (DOY 155), and the grey shaded regions signify the dates that leaves were sampled and measured. The final measurement date was substantially longer than the first two (two-weeks vs. one) due to blocks reaching the late-reproductive (R6) stage at variable times.
Figure 3.S7. Relationship between (A) intrinsic water-use-efficiency ($A_N/g_s$) and the ratio of mesophyll to stomatal conductance to CO$_2$ ($g_m/g_{s-co2}$), (B) $A_N/g_s$ to stomatal conductance to CO$_2$ ($g_{s-co2}$), and (C) $g_m/g_{s-co2}$ to $g_{s-co2}$. Data are from the eight soybean cultivars grown as part of the field experiment and measured at three growth stages indicated by symbol shading. Different symbols represent different cultivars. Coefficients of determination ($\Omega^2_0$) and regression lines are from linear mixed models with the x-variable and growth stage as predictors, and cultivar treated as a random effect. In A, the partial correlation ($r_{xy|z}$) of $A_N/g_s$ with $g_m/g_{s-co2}$ after accounting for $g_{s-co2}$ is also presented.
Figure 3.S8. Relationships between mesophyll conductance ($g_m$) and (A) leaf mass per area ($LMA$), (B) leaf thickness ($L_T$), (C) leaf density ($L_D$), and (D) leaf dry matter content ($LDMC$) from the field experiment. The three growth stages measured were late-vegetative (V4-V5, black symbols), early-reproductive (R2-R4, dark grey), and late-reproductive (R6, light grey). Different symbols represent different cultivars. Coefficients of determination ($\Omega^2_0$) and regression lines are from linear mixed models with the x-variable and growth stage as predictors, and cultivar treated as a random effect.
Table 3.S1. Comparisons of standardized major axis regression slopes from primary bivariate relationships presented for all possible comparisons of the three growth stages (Late-Veg., Early-Rep., and Late-Rep.) measured on field-grown plants and those grown in controlled growth chambers (Chamber). Pair-wise differences in slopes were assessed by likelihood ratio tests with significance corrected for multiple comparisons using the Sidak correction. Comparisons with differing slopes are indicated: * indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

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Table 3.S2. Cultivars used in this study along with their maturity group status and the source of seeds. Abbreviations used on figure axes and in legends are included for clarity. MG is soybean maturity group.

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*Designates cultivars used for field plantings. United States Department of Agriculture, Agricultural Research Service, National Genetic Resources Program. Mineral, Virginia, USA. Westfield, Indiana, USA. Orville, OH, USA.
Figure 4.S1. On-farm yield trend for U.S. soybean, 1924-2015. Data from the U.S. Department of Agriculture, National Agriculture Statistical Service (https://www.nass.usda.gov/Quick_Stats/).
Figure 4.S2. Example trace of chlorophyll a fluorescence induction kinetics over the first two seconds of leaf illumination. Maximum fluorescence ($F_m$) is marked here with the second vertical blue line. The area between the two vertical blue lines and under the trace is the $OJIP_{\text{Area}}$ and is illustrative of photochemical performance. The K-step, which is indicative of heat stress, is the relative fluorescence at 300 $\mu$sec after illumination and is labeled where the red line touches the fluorescence trace. Note that the x-axis is on a log-scale.
Figure 4.S3. Example of thermal (A) and visible (B) image pairs of field plots. In B, the dry (1) and wet (2) references, and plot identification card (3) are labeled. The visible images were used to identify leaves that were clearly sunlit for extraction of temperatures in the paired thermal image.
Figure 4.S4. Maximum carboxylation capacity (A; $V_{C\text{max}}$) and maximum electron transport capacity (B; $J_{\text{max}}$) by year of cultivar release. Each trait was estimated at the late-vegetative (black symbols) and mid-reproductive (red symbols) growth stages.
Figure 4.S5. Stomatal conductance to water vapor (A; $g_{sH}$) and mesophyll conductance (B; $g_m$) by year of cultivar release. Each trait was estimated at the late-vegetative (black symbols) and mid-reproductive (red symbols) growth stages.
Figure 4.S6. Predawn leaf water potentials ($\Psi_{pd}$) by cultivar year of release measured on four days of the year (DOY) indicated by symbol color. The least squares regression fit was not significant ($p=0.062$).
Figure 4.S7. Leaf area index (LAI) by year of cultivar release.