

Examining the Mechanisms by which Photosynthetic Capacity and Water Use Efficiency are Regulated in Wheat Exposed to Soil Drying

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Abstract:

Wheat contributes significantly to human nutrition and livelihoods around the world, but is highly susceptible to drought stress, which is expected to become more prevalent as the climate changes. Therefore, it is increasingly important to assess wheat genotypic variability for tolerance to water deficit and design screening techniques for use in breeding programs. However, genetic variation in whole plant water use efficiency (WUE_{wp} , - biomass per water used) is not always correlated with variation in leaf level water use efficiency (WUE_i - assimilation per stomatal conductance), increasing the difficulty of phenotypic prediction. To understand the disconnect, a mix of spring wheat cultivars and landraces from the Watkins collection were examined for variation in the mechanisms regulating biomass gain (BM) and water use (WU) as components of WUE_{wp} . Specifically, the impact of leaf age and soil drying on stomatal conductance (g_s) and assimilation (A) were assessed. Significant variation was observed for WUE_{wp} (two-fold), with genotypes Krichauff and G1 (Watkins) consistently displaying high WUE_{wp} and Gatsby low WUE_{wp} . Increased WUE_i was correlated with increased WUE_{wp} , even though no significant genetic variation was observed for WUE_i . Additionally, sustained A across leaf age was observed in Krichauff but not Gatsby, corresponding with their measures of WUE_{wp} . Further, lower levels of photosynthetic limitation by rubisco (V_{cmax}) and decreased leaf biomass partitioning (LP) were correlated to higher WUE_{wp} . While WUE_i may not always predict variation in WUE_{wp} , other variables (V_{cmax} , LP, and sustainability of A across leaf age) were strongly associated with the whole plant response. Thus, in vivo measures of photosynthetic limitation and other whole plant proxies, such as area-based estimates of leaf partitioning, serve as useful tools in predicting WUE_{wp} .

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Declaration:

I declare that the work contain herein is original and my own and has not been submitted in this form or any other for the award of a higher degree elsewhere

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Abbreviations:

Measure	Abbreviation
Biomass gain	BM
Water use	WU
Whole Plant Water Use Efficiency	WUE _{wp}
Carbon Assimilation / Photosynthesis	A
Stomatal Conductance	g_s
Intrinsic Leaf Level Water Use Efficiency	WUE _i
Intercellular CO ₂	C_i
Leaf Biomass	LB
Leaf Biomass Partitioning	LP
Leaf Area	LA
Leaf Water Potential	Ψ_L
Relative Water Content	RWC
Gravimetric Water Content	GWC
Abscisic Acid	ABA
1-Aminocyclopropane-1-Carboxylic Acid	ACC
Ribulose -1, 5-bisphosphate carboxylase/oxygenase	Rubisco
Foliar abscisic acid content	[ABA _i]
Maximum carboxylation rate of Rubisco	V_{cmax}
Maximum rate of ribulose-1, 5-bisphosphate regeneration	J_{max}
Vapour Pressure Deficit	VPD
Well-Watered	WW
Water Deficit	WD

Introduction:

The implications of decreased global water availability have received expanding attention in recent decades, as water plays a central role in food security. Many areas are witnessing a decline in agriculturally useful water via reductions of water table levels and changes in rainfall and temperature patterns. Depletion of below ground water resources has been attributed to intensified water use due to both population growth and increased irrigation (e.g. the North China Plain) (Ringler and Zhu 2015, Sun et.al. 2010, Shu et.al. 2012). It is estimated that from 1960 to 2010, 70 % of increased global water use was attributed to a rise in water consumed for irrigation (Wada and Bierkens 2014). Thus, it is important to focus research efforts on developing crops with improved water use efficiency (WUE), identifying varieties that accumulate biomass and use water more efficiently. These varieties will be useful in regions responsible for producing staple grains that are experiencing water shortage, resulting in unsustainable use of water resources (Chen et. al. 2018). Wheat is among these staple grains, and is used for both human and animal consumption, leading to a complex relationship with food security and resource management.

In wheat, many research groups have focused on understanding the causes of genetic variation in WUE. At the whole plant level, WUE_{wp} is defined by either the biomass per the amount of water transpired (WUE_{bwp}) or the yield per amount of water transpired (WUE_{ywp}) (Leakey et.al. 2019). Alternatively, WUE can be defined intrinsically (WUE_i), as carbon assimilation rate per stomatal conductance. WUE_i is easily and efficiently measured with infra-red gas analyzers (e.g. LICOR systems described below) but is infrequently correlated to WUE_{wp} (Gilbert et.al. 2011, Flexas et. al. 2016, Leakey et.al. 2019). As such, it is crucial to identify how intrinsic measures may be improved to more accurately predict the whole plant response.

A variety of mechanisms have been proposed for linking WUE_{wp} and WUE_i , where these mechanisms regulate WUE by altering water consumption and/or net carbon gain (Leakey et.al. 2019, Medrano et.al. 2015, Medrano et.al. 2012). For example, decline in stomatal conductance restricts transpiration (water use) and CO_2 intake (carbon gain) which, dependent on the magnitude of change, will have a positive or neutral impact on WUE (Leakey et. al 2019). Alternatively, improvements in the biochemical efficiency of photosynthetic enzymes improve carbon gain without changing water use (Reynolds et.al. 2009, Parry et.al. 2010, Carmo-Silva 2015, Flexas et.al. 2016). In wheat, genetic variability has been noted for many of these traits (Carmo-Silva et.al. 2015, Carmo-Silva et.al. 2017, Driever et.al. 2014) although it is less clear how they interact under water deficit. The outlined project aimed to identify the mechanisms leading to differences in WUE_{wp} and WUE_i , and how they may co-vary under water deficit and across leaf age. To do so, mechanisms that regulate carbon gain and water use were assessed. These included examining the regulation of assimilation by rubisco efficiency and of stomatal conductance by the phytohormones abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC)

Literature Review:

Water use efficiency (WUE) has been examined extensively in wheat as it is one of the most widely consumed crops, accounting for approximately 20 % of the world's calories (Reynolds et.al. 2009). Thus, it is necessary to prioritize improving WUE under global water scarcity. Many studies have focused on screening wheat and its relatives for variation in response to water deficit both at the whole plant and leaf level. These works have indicated differential adaptation of wheat genotypes to water deficit, represented by increased or maintained WUE under water limited conditions (Wu and Bao 2011, Scotti-Campos et. al. 2015, Veneklaas and Peacock 1994).

Wheat WUE response to water deficit can be examined based on the whole plant response (WUE_{wp}) or the intrinsic response (WUE_i) as both indicators serve as valuable tools for discerning genotypic variation under water deficit. WUE_{wp} , was calculated here as above the ground biomass gained (BM) per water consumed during the treatment period. Additionally, WUE_i was calculated as carbon assimilation (A) per the stomatal conductance to H_2O (g_s). The two WUE indicators are defined below.

$$WUE_{wp} = \frac{BM}{WU} \quad WUE_i = \frac{A}{g_s}$$

Genotypic variation exists in wheat for WUE_i , WUE_{wp} , and their components under water deficit conditions (Akhkha et. al. 2011, Scotti-Campos et.al. 2015). Additionally, tradeoffs exist between and within these relationships. Stomata close under water deficit, which in turn decreases plant water use via transpiration but also decreases CO_2 available for carbon assimilation and biomass gain (Gilbert et.al. 2011, Leakey et.al. 2019). Further, plants with greater biomass under water deficit have resultingly higher levels of transpirable leaf area and water use (Leakey et.al. 2019, Borrell 2014). Thus, it is necessary to identify varieties that balance water use and carbon gain under low water status and examine variability for their regulatory mechanisms. These leaf level and biochemical mechanisms exhibit genetic variation and may serve in adapting measurements of WUE_i to better predict WUE_{wp} . It is therefore important to understand how water deficit impact plants at the whole plant, leaf, and biochemical levels.

Impact of Water Deficit on WUE_{wp} and Whole Plant Level Traits

Whole plant traits are measured with low-tech, low through-put methods and are often significantly altered by water deficit. These traits include leaf area, total biomass, biomass partitioning, and hydraulic conductance. Biomass accumulation (BM) and total water use (WU) are restricted under water deficit where the magnitude of decrease varies between genotypes (Wu and Bao. 2011, Changhai et. al. 2010, Tartar et. al 2015, Veneklaas and Peacock. 1994). Further, WUE_{wp} , varies between genotypes in multiple crops including wheat (Changhai et. al. 2010, Tartar et. al 2015, Veneklaas and Peacock. 1994) due directly to variation in BM and WU. Response of WUE_{wp} to water deficit differs between species, showing both increased (Ryan et. al 2016) and unchanged levels (Leakey et.al. 2019) under low water availability. Such responses enhance the importance of identifying

how changes in WUE_{wp} correlate to changes in WU and BM between genotypes and treatments.

Further, optimizing biomass partitioning has been suggested as a method for improving WUE_{wp} and drought tolerance in wheat as it is shown to be unchanged by water deficit (Veneklaas and Peacock 1994). Maintaining proportional biomass partitioning ensures that leaf area can sustain photosynthesis for grain fill without excess water use early in the growing season (Borrell et.al. 2014). However, known drought tolerant and high WUE_{wp} wheat varieties have been shown to reduce their leaf area less than their intolerant counter parts (Wu and Bao 2011), maintaining photosynthetic area at the expense of water use. Transpiration in response to soil moisture is regulated by hydraulic conductance and leaf water potential, as these determine the ability and demand of water to be drawn across the plant water potential gradient (Shatil-Cohen et.al. 2011, Pantin et.al. 2013). Thus, increased transpiration via increased leaf area or vapour pressure deficit (VPD) leads to increased water use, unless an alternative restrictive mechanism exists.

WUE_{wp} and other whole plant traits show evident genetic variation which is achieved by the cumulative response of leaves and other plant organs (Medrano et. al. 2015). Measuring whole plant response to water deficit is an essential starting point for identifying underlying regulatory mechanisms by determining whether water use or carbon gain components are impacted. These mechanisms exist at the leaf level and are sensitive to environmental factors including soil water deficit. Leaf level measurements are instantaneous, and environmental variation increases the disconnect between their measure and the whole plant phenotype (Leakey et.al. 2019, Gilbert et.al. 2010, Medrano et.al. 2015, Medrano et.al. 2012). As such, the relationship between leaf level measures, the environment, and the whole plant response needs to be clearly defined.

Impact of Water Deficit on WUE_i and Leaf Level Characteristics

WUE_i and other leaf level traits often require high-tech, high throughput methods of measurement, like quantification of carbon assimilation (A) and stomatal conductance (g_s) with infra-red gas analyzers. Such equipment makes leaf level traits easy to assess and improves phenotyping efficiency. Many of these leaf level characteristics vary across soil moisture and genetic backgrounds, including photosynthetic rate (A), stomatal conductance (g_s), intercellular carbon content (C_i), leaf water potential (Ψ_L), and WUE_i . These traits contribute to variation in WUE_{wp} by regulating BM and WU, however, WUE_i shows no consistent variation in wheat aside from increasing under water deficit (Wu and Bao. 2011, Scotti-Campos et.al. 2015, Changhai et.al. 2010, Zhang et.al. 2018). Increased WUE_i under water deficit is attributed to a more rapid decline in g_s compared to A , where A is restricted by g_s , indicating that WUE_i is largely regulated by stomata (Scotti-Campos et.al. 2015, Wu and Bao. 2011, Gilbert et.al. 2010, Gonzalez et.al. 2010, James et.al. 2002).

Variation in A and g_s has been observed between genotypes in many species including wheat (Wu and Bao 2011), barley (González et.al. 2010), and grapes (Medrano et.al. 2015). Changes in A and g_s are due to changes in both C_i and Ψ_L . Soil water deficit

decreases Ψ_L and leaf relative water content (RWC) (Pantin et.al 2013, Shatil-Cohen et.al 2011, Scotti-Campos et.al. 2015, Li.et.al. 2017b), leading to loss in guard cell turgor, stomatal closure, and decreased water use. The same stomatal closure restricts the amount of CO_2 entering the intercellular space (C_i), limiting the substrate available for photosynthesis and biomass gain (Jauregui et.al. 2018, Wu and Bao 2011, Atkinson et.al. 1989).

Leaf level traits are sensitive to a range of environmental conditions aside from water deficit. High temperature and vapour pressure deficit (VPD) often coincide with low soil moisture and cause unexpected variation in leaf level characteristics. For example, stomatal conductance is more sensitive to changes in VPD under water deficit conditions than under optimal water status (Xue et.al. 2004). Photosynthetic machinery is also susceptible to permanent impairment when exposed to high temperature or prolonged water deficit, hindering carbon assimilation regardless of soil water status (Perdomo et.al. 2017, Lawlor 2002). A and g_s also show differences across the plant canopy due to microclimate and stomatal patchiness, where differences in VPD and light interception cause variation in both measures (Medrano et.al. 2015, Lawson et.al. 1998, Eckstein et.al. 1998, Terashima et.al. 1988, Mott 1995, Buckley et.al. 1999). The sensitivity of WUE_i to environmental conditions, exacerbates the disconnect to WUE_{wp} by altering the cumulative contribution of leaves to the whole plant response. WUE_i declines as leaves age as A decreases more than and g_s (Atkinson et.al. 1989), further indicating that each leaf differentially contributes to biomass gain and water use across time and space. Thus, it is imperative to identify sources of variation when measuring WUE_i , to maximize correlation to WUE_{wp} .

Despite the limitations to measures of WUE_i , it is still a useful tool to further examine the impact of stress on plant productivity as its components, A and g_s , are linked to BM and WU respectively. WUE_i also serves as an intermediary between biochemical mechanism and the whole plant phenotype, as these mechanisms regulate A and g_s . As such, it is worth examining how these biochemical mechanisms are impacted by water deficit and leaf age and if they co-vary with leaf level and whole plant measures. These biochemical components include both phytohormones and photosynthetic enzymes.

Phytohormones, WUE, and Water Deficit

Phytohormones regulate various plant stress responses. Abscisic acid (ABA) increases in leaves of multiple plant species during low water availability, inducing stomatal closure and restricting water use (Li et.al. 2017b, Valluru et.al. 2016, De Ollas and Dodd 2015, Chen et.al. 2013, Speirs et.al. 2013, Sauter, Davies, and Hartung 2001, Saradadevi et.al. 2017, Saradadevi et.al. 2014). In studying plant response to water deficit, it is critical to understand the impact of mechanistic changes induced by enhanced foliar ABA concentrations ($[\text{ABA}_l]$) on WUE_i and WUE_{wp} .

Soil water deficit is perceived by the root tissues as a decline in soil water potential (Ψ_m), resulting in a decline in root water potential (Ψ_r) (Zhang and Davies 1989, Li et.al. 2017b). Subsequent ABA biosynthesis results in increased root ABA content ($[\text{ABA}_r]$) (Zhang and Davies 1989, Sauter, Davies, and Hartung 2001, Wilkinson and Davies

2002). ABA serves as a root to shoot signal that is translocated via xylem as indicated by increases in ABA content in shoot xylem sap ($[ABA_{xs}]$) (Zhang and Davies 1989). The increased $[ABA_{xs}]$ either directly or indirectly increases leaf ABA content ($[ABA_l]$), inducing stomatal closure (Zhang and Davies 1989, McAdam and Brodribb 2016, Wilkinson and Davies 2002).

Guard cells perceive the phytohormone via a complex signaling pathway, resulting in stomatal closure (Described in Klingler et.al 2010, Yin et.al. 2013, Yang et.al. 2017). When foliar ABA is sourced from the roots, stomatal closure is a product of chemical signaling. Alternatively, hydraulic signaling pathways can alter hydraulic conductivity and Ψ_L to induce ABA production directly in the leaf where it can induce stomatal closure (Wilkinson and Davies 2002, Wilkinson and Davies 2010, Comstock 2001, Munns et.al. 1988, Kudoyarova et. Al. 2011). Some have proposed that ABA in the xylem restricts lateral flow of water from the shoot to the leaf through aquaporins in the vascular bundle sheath (Shatil-Cohen et.al. 2011, Pantin et.al.2013, Sade et.al. 2014). Such restrictions would decrease Ψ_L , resulting in increased local ABA production or direct loss of guard cell turgor, subsequently inducing stomatal closure (Pantin et.al. 2013). However, it has been suggested that Ψ_L must drop to levels consistent with severe water deficit to trigger leaf ABA biosynthesis (Sauter, Davies and Hartung 2001). This response varies between species where some rapidly increase ABA levels in response to smaller changes in Ψ_L (McAdam and Brodribb 2016). Further, in wheat, stomatal sensitivity to ABA declines as leaves age without altering $[ABA_l]$ (Atkinson et.al. 1989, Chen et.al. 2013). Additionally, wheat has shown genotypic variation in both $[ABA_l]$ under water deficit and the stomatal sensitivity of ABA (Saradadevi et.al. 2017, Saradadevi et.al. 2014). The pathways by which ABA acts under stress are complex, but increased foliar ABA ultimately decreases g_s , resulting in a corresponding decline in A . However, ABA has no direct effect on A (Terashima et.al. 1988, Mott 1995, Eckstein et.al. 1998). Still, as g_s regulates transpirational water loss, it is then important to examine variation in $[ABA_l]$ and stomatal sensitivity to ABA when studying the mechanisms underlying WUE_i and WUE_{wp} . The details of the production and perception of ABA are outside the scope of the present study, but it is worthwhile to consider the interactions that occur between ABA and other plant hormones under stress.

It is unlikely that ABA is the only hormone involved in plant perception of water deficit, as exogenous application of ABA in quantities consistent with the endogenous content do not elicit the same leaf level response (Saradadevi et.al. 2014, Munns et.al. 1988) However, the role of other phytohormones is less well understood. One phytohormone of interest is Ethylene and its precursor 1-Aminocyclopropane -1-Carboxylic Acid (ACC), due to its involvement in “cross talk” with ABA. ABA and ethylene/ACC modulate each other’s effect in plant stress response via alteration in perception or production, where ethylene restricts ABA induced stomatal closure (Sharp and LeNoble 2002, Wilkinson and Davies 2009, Wilkinson et.al. 2012, Tanaka et.al. 2005). Further, ethylene/ACC regulate shoot and leaf growth and leaf senescence under water stress (Sharp and LeNoble 2002, Sobeih et.al. 2004, Chen et.al.2013, Wilkinson and Davies 2010, Young et.al. 2004). ACC content of plant tissue is used as a proxy for ethylene gas evolution (Bulens et.al. 2011), as it is the hormone’s precursor and more directly indicates the plant

stress response. ACC is thought to be synthesized in roots exposed to stress and translocated to the shoot where it is converted to ethylene (Wilkinson and Davies 2010).

ACC or ethylene levels increase in tomatoes exposed to salinization and water deficit (Ghanem et.al. 2008, Sobeih et.al. 2004) as well as in droughted maize (Li.et.al. 2017b, Young et.al. 2004) and wheat leaves (Yang et.al. 2014). In wheat, ethylene increase correlated with a decrease in A (Yang et.al. 2014), and ACC or ethylene-induced stomatal closure increased as leaves aged with no change in foliar content (Chen et.al. 2013). Contrastingly, increases in ethylene evolution (via ethephon spray) correlated with increases in g_s and A of mustard plants, suggesting species specificity (Iqbal et.al. 2011). Nevertheless, chemical inhibition of wheat ethylene evolution caused earlier stomatal response to water deficit, induced stomatal closure in well-watered plants, and increased ABA production (Sharipova et.al. 2012). Additionally, chemical inhibition of stress-induced ethylene evolution resulted in an increase in both A and g_s , and higher Rubisco and chlorophyll content (Yang et.al. 2014, Young et.al. 2004). These findings indicate an inconsistent relationship between ACC/ethylene and parameters of WUE_i , although some impact is evident. In wheat, the effect of ACC/ethylene varies between well-watered and water deficit conditions (Sharipova et.al. 2012), which may be linked to variation in ABA content. Thus, it is of interest to quantify both ACC and ABA under stress and examine how the balance of the two may induce mechanistic changes such as stomatal closure or photosynthetic decline.

Photosynthetic Capacity and Limitations under Water Deficit

Increased photosynthetic capacity is important in achieving improved WUE_i , as it is linked to increased carbon assimilation (A). Measurements of photosynthesis can be made via *in vitro* or *in vivo* methods, where both have detected variation across plant development, organ age, and measurement conditions (Gilbert et.al. 2011, Prins et.al. 2016, Carmo-Silva et.al.2016, Jauregui et.al. 2018). These measurements contribute to our understanding of how leaf level traits relate to whole plant responses, with each serving a distinct purpose.

Instantaneous *in vivo* measurements provide the best approximation of a leaf's photosynthetic response to a given environment. These are commonly measured using portable infrared gas analyzers, like those provided by LICOR bioscience (Lincoln, Nebraska USA), that are easy and relatively efficient to use. Such equipment serves as an option for improving plant phenotyping and selection efficiency, by measuring several parameters associated with enhanced yield and stress tolerance (e.g. stomatal conductance, chlorophyll fluorescence, and net carbon gain).

Measurement of a leaf's net carbon gain (or assimilation) can approximate leaf photosynthetic capacity in that moment, mirroring the relative contribution to biomass gain. However, as previously noted, localized momentary measurements infrequently correlate to the whole plant response and are complicated by water deficit (Gilbert et.al. 2011, Leakey et.al. 2019, Medrano et.al. 2015). Specifically, levels of stomatal closure experienced under severe water deficit increase the difficulty of predicting intercellular CO_2 concentration (C_i) which is necessary for calculating assimilation (Gilbert et.al.

2011). Additionally, stomatal aperture has been noted to vary across leaves as soil dries indicating patchy stomatal behavior (Lawson et.al. 1998, Eckstein et.al. 1998, Terashima et.al. 1988). Stomatal patchiness leads to variation in A and C_i across the leaves and thus difficulties in accurate predictions of total C_i (Terashima et.al. 1988, Mott 1995, Buckley et.al. 1999). Further, such *in vivo* measures of photosynthesis may be influenced by interaction with other parameters. Decreased stomatal conductance (g_s) limits carbon assimilation (A) by restricting C_i and substrate available for photosynthesis. Such limitations to carbon assimilation are referred to as stomatal-limitations and are easily determined by comparing the photosynthetic response of water deficit plants to their well-watered counterparts (Lawlor 2002, Gilbert et.al. 2011, Wu and Bao 2011, Luo et.al. 2016). Assimilation may also be limited by non-stomatal factors, such as decline in the efficiency of CO₂ fixation into the plant by the enzyme Ribulose -1, 5 -biphosphate carboxylase/oxygenase (Rubisco) (Lawlor 2002, Gilbert et.al. 2011).

Both stomatal and non-stomatal/biochemical limitation may be predicted from the relationship between A and C_i , in the form of “ A/C_i ” response curves (Lawlor 2002). These are fitted through *in vivo* measurements of A and C_i at increasing levels of CO₂ ranging from 50 ppm to 1000 ppm (or greater). As CO₂ increases both A and C_i increase until saturation is reached, after which no gain in A may be achieved regardless of increase in C_i . Under optimum water and saturating light conditions (i.e. no environmental limitations to A or g_s) this point of saturation is termed maximum assimilation (A_{max}), and indicates non-stomatal limitations imposed by the carboxylation efficiency of Rubisco (Lawlor 2002). Under water deficit induced stomatal closure, this level of saturation is reduced and is termed the potential assimilation (A_{pot}) (Lawlor 2002). Reductions in A_{pot} may be permanent or reversible and have shown genetic variability in cotton and wheat (Wu and Bao 2011, Luo et.al. 2016). Reversible limitations to A_{pot} are largely due to stomatal limitation and can be counter-acted by restoring optimum soil moisture and stomatal conductance (Lawlor 2002). Alternatively, permanent non-stomatal limitation cannot be restored upon re-watering and indicates metabolic inhibition by the photosynthetic machinery (Lawlor 2002). Although notably, the aforementioned patchy stomatal behavior may cause false indications of non-stomatal limitation from A/C_i curves due to over prediction of values of C_i (Mott 1995). Knowing which form of limitation has occurred indicates the extent photosynthetic inhibition by water deficit in both the short and long term. Such knowledge may assist with varietal selection, as genotypes with less stomatal limitation and no non-stomatal limitation in response to water deficit would be preferred.

A/C_i response curves also predict differences in the biochemical mechanisms underlying the observed photosynthetic response. Both the maximum carboxylation rate of Rubisco (V_{cmax}) and the rate of ribulose-1, 5-biphosphate regeneration (J_{max}) may be determined from the response curves via the models of Farquhar et.al. 1980 (Gilbert et.al. 2011, Flexas et.al. 2016). These parameters vary between genotypes in soybean (Gilbert et.al. 2016) and have been highly correlated to A_{max} in field grown wheat (Carmo-Silva et.al. 2016). Thus, V_{cmax} and J_{max} can aid varietal selection and are important in examining tolerance to water deficit.

In vitro measurements provide validation and further explanation of *in vivo* measurements. These are performed as assays on tissues measured for *in vivo* photosynthesis and examine parameters underlying the response, such as the carboxylation activity and content of Rubisco, and the content of chlorophyll and total soluble protein (TSP). Such connections are valuable as sample measurements at the leaf level cannot always predict the whole plant response. Measuring the associated biochemical parameters confirms the leaf level response and helps us to adapt the timing and execution of *in vivo* measurements, thus improving their correlation to the whole plant phenotype. However, leaf level measurements of photosynthetic limitation as detailed above are highly correlated to their biochemical counter parts (Carmo-Silva et.al. 2016). While these *in vitro* measurements may not be necessary, they help to determine the impact of the environment and organ age on photosynthetic biochemistry.

Here, the importance of whole plant, leaf level, and biochemical measures in WUE phenotyping has been highlighted. These parameters are related to crop productivity and frequently interact and respond to stress. Genetic variability has been detected for a number of these traits in wheat including photosynthetic rate, stomatal conductance, WUE_{wp} , enzyme efficiency, and hormone sensitivity. Understanding how these components are related and correspond under stress will enhance our ability to identify superior crop varieties. Thus, it is crucial to conduct work to characterise the response of these parameter to factors such as leaf age and water deficit to bridge the disconnect between WUE_i and WUE_{wp}

The work presented here aimed to determine the underlying mechanisms that regulate the relationship between WUE_i and WUE_{wp} , and whether water deficit and leaf age alter these mechanisms. The magnitude of variation in wheat for WUE_{wp} was quantified by exposing a mix of landraces (Watkins collection – Wingen et.al. 2014) and commercial cultivars to water deficit. Focus was placed on the limitation of A by inherent photosynthetic biochemistry as well as on the regulation of g_s via phytohormones (ABA and ACC) across leaf age and soil drying. Soil water deficit was hypothesized to limit A through both stomatal and non-stomatal means, with the magnitude of each varying between genotypes. Stomatal limitations were examined through the relationship between g_s and gravimetric water content (GWC) where the rate of stomatal closure across soil drying was expected to vary between genotypes. Non-stomatal limitation was determined by the rebound of A upon re-watering and through the fitting of A/C_i curves where metabolic inhibition was expected to increase following water deficit. Further, stomatal sensitivity to ABA and ACC was examined, where stomatal conductance was predicted to vary as a result of changes in the balance between these hormones as leaves age or soil moisture declines. These mechanisms would help explain momentary variation in measures of A , g_s , and WUE_i and how they relate to the whole plant phenotype.

Materials and Methods:

Plant Materials

Spring wheat genotypes from the Watkins collection (Wingen et.al. 2014) as well as modern industry cultivars were used. The lines were selected for their previously noted response to water deficit as described below.

Drysdale (“DR”)

Drysdale is a modern Australian cultivar that has high expression of the TaER gene which has been linked to improvements in photosynthetic capacity during water stress (Zheng et.al. 2015). It has shown high WUE_i, high photosynthetic rate, and low carbon isotope discrimination (Zheng et. al. 2015, Schoppach and Sadok. 2012). It also demonstrated an earlier change point in response to increasing VPD when compared to other elite cultivars (Schoppach and Sadok 2012).

Gatsby (“GA”)

Gatsby has previously shown increased photosynthetic rates despite low levels of Rubisco and is a modern industry cultivar from the UK (Carmo-Silva et.al. 2017)

Krichauff (“KR”)

Krichauff is a modern Australian cultivar and is adapted a dry climate. It has previously been included as a high WUE check against Australian landraces G64 and G1. Krichauff had a linear transpiration response to increasing VPD (Schoppach and Sadok 2012) but closed its stomata earlier in response to soil drying

Paragon (“PA”)

Paragon is a modern industry cultivar from the United Kingdom (UK). It was previously crossed with members of the Watkins collection (Wingen et.al. 2014) and is a common check cultivar in experiments on wheat

Watkins line G1

G1 is an Australian land race that responded near or at the level of Drysdale for total plant dry mass and leaf partitioning when exposed to varying levels of soil drying (Marshall 2018). Other responses that are worth noting include low stomatal conductance and chlorophyll content under water deficit (Marshall 2018).

Watkins line G57

G57 displayed similar response to Paragon for harvest index and above ground biomass when exposed to soil drying (Onate – BBSRC report 2018). Total grain weight and ABA content were higher in G57 compared to Paragon (Onate – BBSRC report 2018).

Watkins line G64

G64 is an Australian land race that responded similarly to Drysdale for total plant dry mass and leaf partitioning when exposed to varying levels of soil drying (Marshall 2018). Other responses that are worth noting include higher chlorophyll content and higher stomatal conductance under well-watered conditions (Marshall 2018).

Watkins line G83

G83 was selected per the suggestion of a colleague (Cristina Sales – Personal Communication October 2018) due to its interest in studies concerned with improved photosynthetic capacity.

Watkins line G91

G91 was selected per the suggestion of a colleague (Cristina Sales – Personal Communication October 2018) due to its interest in studies concerned with improved photosynthetic capacity.

Genotypes Used in Each Experiment				
<i>Experiment</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>
<i>Genotypes used</i>	<i>All</i>	<i>G1</i>	<i>G1</i>	<i>G1</i>
		<i>G53</i>	<i>G83</i>	<i>GA</i>
		<i>G91</i>	<i>GA</i>	<i>KR</i>
		<i>GA</i>	<i>KR</i>	
		<i>KR</i>		

Table 1: Examined genotypes differed in each experiment as detailed above. Selections were made as detailed in the results section



KR G83 GA G1

Figure 1: Genotypes used in experiment 3, where they entered reproductive stage.

<i>Growth Conditions by Experiment</i>				
<i>Experiment</i>	1	2	3	4
<i>Sowing Date</i>	Oct 24, 2018	Jan 16, 2019	April 15, 2019	May 8, 2019
<i>Harvest Date</i>	Dec 8 - 10, 2018	Feb 26, 2019	June 23, 2019	June 23, 2019
<i>Duration</i>	42 days	42 days	69 days	46 days
<i>Date of treatment</i>	Nov 16, 2018	Feb 10, 2019	1: May 6, 2019 2: May 23, 2019	May 29, 2019
<i>Treatment</i>	Water deficit	Water deficit	1: Leaf age 2: Water deficit	Leaf age
<i>Location*</i>	GH 8	GH 10 and 14	GH 14	CE 9
<i>Number of Plants</i>	108	100	72	72
<i>Average humidity</i>	40.2 %	34.0 %	48.8 %	59.3%
<i>Min temperature</i>	16.7 °C	15.5 °C	19.1 °C	18.6 °C
<i>Max temperature</i>	28.6 °C	30.1 °C	27.4 °C	24.3 °C
<i>Light Range**</i>	Radiation (PAR) 48 – 360 $\mu\text{mol}/\text{m}^2/\text{s} \pm 50$	Radiation (PAR) 48 – 360 $\mu\text{mol}/\text{m}^2/\text{s} \pm 50$	Radiation (PAR) 48 – 360 $\mu\text{mol}/\text{m}^2/\text{s} \pm 50$	Spectral Radiation (400-700nm) 200 $\mu\text{mol}/\text{m}^2/\text{s} \pm 30$
<i>Pre-germination treatment***</i>	Yes	Yes	No	No

Table 2: Environmental conditions and timeline for each experiment. Meaning of asterisk symbols are indicated below.

* GH = glasshouse, CE = controlled environment room

** GH measure = Macam Q203 Quantum Radiometer by Irrdian Ltd, Tranent, Scotland

CE measure = PG100N Spectrometer made by UPRTek Europe, Aachen, Germany

*** Pre-germination treatment is described below in “plant culture”

Plant Culture

Conditions were set to 16-hour / 24°C days and 8-hour / 18°C nights. Glasshouses received supplemental lighting below 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with sodium lamps (600Watt Plantastar made by Oram Ltd, Newton-Le-Willows, UK), and CE room 9 received 16 hours of LED lighting (B150 NS1 made by Valoya Oy, Helsinki, Finland). A mix of 3:1 Petersfield compost to Silver Sand was used as a growth medium, as it has been optimized for glasshouse grown wheat. Two-liter pots were filled and subsequently saturated until water dripped from the pots at which point seeds were sown. Pots drained overnight (from 17:00 to 9:00) and were weighed to determine weight at drain capacity. Pots were randomly assigned to a genotype, treatment, and replication using the EDGAR tool (developed by James K. M. Brown, Cereals Research Department, John Innes Centre - Norwich, England). Prior to sowing for experiments 1 and 2, seeds were exposed to 4°C for 24 hours to improve germination rate. In experiments 3 and 4, no pre-germination treatment was applied and no impact on germination rate was observed. Two - four seeds per pot were sown, and then thinned at 1-week post emergence to 1 seedling per pot. Growth locations, duration, and conditions varied between experiments as outlined in Table 2.

<i>Measurements Conducted by Experiment</i>				
<i>Experiment</i>	1	2	3	4
<i>Stomatal Conductance (porometer)</i>	Y	N	N	N
<i>Leaf Length</i>	Y	N	N	N
<i>Leaf Area</i>	Y	Y	N	N
<i>Biomass</i>	Y	Y	Y	Y
<i>Partitioning</i>	Y	Y	N	Y
<i>Water Use</i>	Y	Y	Y	Y
<i>Gravimetric water content (soil)</i>	Y	Y	Y	Y
<i>Leaf water potential</i>	Y	Y	N	N
<i>Relative water content</i>	N	N	Y	Y
<i>LICOR (sample measurements)</i>	Y	Y	Y	Y
<i>LICOR (A/C_i)</i>	N	N	Y	N
<i>ABA</i>	Y	Y	Y	Y

Table 3: A guide to the measurements conducted in each experiment where Y = yes it was measured and N = no it was not measured

Measurements Common to Multiple Experiments

Stomatal Conductance

Stomatal conductance was measured with an AP4 porometer in experiment 1 (Delta T Devices – Cambridge, UK). Measurements were performed between the hours of 8:00 and 15:00 daily to ensure optimal stomatal responsiveness (Appendix A - Figure A.1). Calibration took place first thing in the morning between 8:00 and 8:30 with a calibration plate that was prepared the previous evening, and no additional calibration was performed throughout the day. To measure the porometer head was clamped on to the youngest fully expanded leaf until the reading was stable (as indicated by the porometer beeping twice). The adaxial side of the leaf was used for measuring, and new leaves were selected once a younger leaf was fully emerged. One leaf per genotype was used, and measurements were taken in the same pattern each day based on the EDGAR randomization. On days of re-watering, measurements were taken both 30 minutes before and after irrigation.

Plant Water Relations

To determine gravimetric water content at harvest and throughout the experiment, soil from de-topped two-liter pots was placed into paper bags and dried in a drying oven at 105°C for at least a week. Soil dry weight was determined as the weight of the bag and soil minus the weight of the bag. Soil saturated weight was determined as the pot and soil weight at maximum drain capacity minus the pot weight. Soil weight at measurement was determined as the pot and soil weight at the time of measurement minus the pot weight. Gravimetric water content was calculated as illustrated below where W_{ds} , W_{ss} , and W_{ms} are the soil dry weight, soil saturated weight, and soil weight at measurement respectively.

$$GWC = (W_{ms} - W_{ds}) / (W_{ss} - W_{ds})$$

Water content at drain capacity was determined by the weight of each pot at soil saturation (after draining overnight) minus the average soil dry weight in two-liter pots, as determined by a preliminary experiment (Appendix A – Table A.1). This water content was determined in grams and was considered 100% of drain capacity. The proportion of drain capacity was determined in 10% increments (i.e. 90%, 80%...20 %) by multiplying the water content at 100% drain capacity by the corresponding decimal values (i.e. 0.9, 0.8...0.2). These values were calculated for each biological replicate and used to re-water plants by administering water to pots on a balance until the reading was within +/- 5 grams of the target weight.

Relative water content was determined by measuring the weight of the leaf tip at sampling (W_{hl}), soaking it in a Falcon tube with DI water overnight (9 hours) to obtain the saturated weight (W_{sl}) and then drying it in the drying cabinet (60°C) for 1 week to obtain the dry weight (W_{dl}). Relative water content was calculated via the equation below:

$$RWC = (W_{hl} - W_{dl}) / (W_{sl} - W_{dl})$$

Water use was determined as the daily change in pot weight minus the average evaporation from bare soil pots. To achieve this, pot weight was logged daily. The cumulative water use was calculated as the sum of the daily water use across the entire experiment.

Biomass and Leaf Area

Fresh biomass was determined by cutting stems at the soil level and weighing the entire above ground portion on a balance. Leaves and stems were separated and weighed to determine fresh weight partitioning ratios. Subsequently, plant material was placed into white paper bags in a drying cabinet at 60°C for 2 weeks when dry weight and partitioning ratios were determined. Partitioning ratios were calculated as the weight of the plant organ of interest (leaf or stem) divided by the total weight.

Biomass gain over the treatment period was determined as the average plant weight at the initial harvest (third leaf stage) minus the weight of each biological replicate at the end of the treatment period.

Leaf area was determined after fresh weight measurements with a LI-COR Model 3100 leaf area meter (LI-COR Bioscience – Lincoln, Nebraska USA). All leaves were placed on the conveyor, ensuring they entered the light plane without folding or distortion to improve accuracy. Leaf area was consistently correlated to leaf biomass, although more time consuming to measure. Therefore, leaf biomass and not leaf area was measured in experiment 3 and 4.

Licor Measurements



Figure 2: Image depicting the LI-6400xt clamped onto a leaf with the fluorometer head attachment

A LI-6400xt was used to measure photosynthetic rate and stomatal conductance and collect A/C_i data. Instantaneous sample measures were conducted between the hours of 8:30 and 13:00 and were carried out in the same pattern at each measurement based on the EDGAR randomization. During instantaneous sample measurements of A and g_s , conditions were set to a flow rate of 300 $\mu\text{mol/s}$, a CO_2 value of 400 ppm, a block temperature of 26°C, and a light level of 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with the leaf fan on high. To measure, the LI-6400xt fluorometer head was clamped onto a leaf (Figure 2) until

stomatal conductance and photosynthesis reached a steady state (approximately 3 to 5 minutes). Once stabilized, 2 to 3 data points were logged per plant, each approximately 5 to 10 seconds apart.

A/C_i data was collected on flag leaves through a similar process. Conditions were set to a flow rate of $300 \mu\text{mol/s}$, a block temperature of 26°C , and a light level of $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ with the leaf fan on high. The initial CO_2 was set at 420 ppm, to approximate ambient levels. At all set points g_s , A , and C_i were allowed to stabilize and then logged twice. Following the ambient set point CO_2 was sequentially decreased in 50 ppm decrements starting at 350 ppm and ending at 50 ppm, logging twice at each point. During this phase it was important to not spend excess time at the lowest points (i.e. 50 and 100 ppm) as prolonged plant exposure to severely suboptimal CO_2 may inactivate Rubisco and reduce photosynthetic capacity, thus rendering curves inaccurate. Once the final lowest CO_2 value was logged, the level was returned to ambient and plants were acclimated back to the initial levels of A and C_i . From here CO_2 was stepped up by 50 ppm, starting at 470 ppm and ending at 1000, logging twice at each point. After the final measurement leaves were removed from the chamber.

Sampling Procedure



Figure 3: Image depicting the razor blade and cork apparatus used to collect leaf samples. The samples taken in the image was the first sample collected from this leaf, 5 cm from the leaf tip

Samples were collected for ABA, Rubisco, and ACC determination at least one hour after gas exchange was measured in the same leaf. Sampling began at least 5 cm from the leaf tip and was conducted using a razor blade and cork apparatus and a cork block (Figure 3) that consistently produced 2.5 cm leaf samples. The samples were dropped into a Dewar dish containing $\text{N}_2(\text{l})$ and placed in pre-labeled 1.5 mL Eppendorf tubes that had been

stored in a bath of $N_2(l)$. Tubes were placed into Dewar flasks while the remaining replicates were sampled. The leaf width of each sample end was determined by measuring the width of the leaf tip and that of all attached cut sites with an electronic caliper. Samples were always collected in the same order, beginning with ABA nearest the leaf tip, then Rubisco, and finally ACC. Upon completion of sampling, the leaf sections were stored in a -80°C freezer until the assay date.

Assays

ABA Radioimmuno Assay

ABA was measured as previously described (Quarrie et al. 1988). After leaf samples were snap frozen in $N_2(l)$, they were freeze dried for 48 hours, then ground in pre-weighed 2 mL Eppendorf tubes with 2 ball bearings using a ball mill (30 shakes/s for 40 seconds). Ball bearings were removed, and tubes were re-weighed to determine the weight of the dried leaf material. DI water was then added to in a 1:50 ratio (sample:water) to extract ABA. These tubes were then placed in a cold shaker for 14.25 hours and then stored back in a -20°C freezer until assays were conducted.

The assay performed was a Radio-Immuno assay, described in detail in Quarrie et.al. 1988. Levels of ABA were determined using a scintillation counter that quantified the samples level of radioactivity (CPM). A set of standards with known concentrations of ABA (0, 62.5, 125, 250, 500, 1000, 2000pg ABA per 50 μL and a saturating dose) was used to fit a calibration curve, from which the level of sample ABA was obtained (Ng of ABA per g of plant dry weight).

Rubisco and ACC assays were not performed as a part of the work presented here.

Statistical methods

Data analysis was conducted using R software. Packages dplyr and tidyr were used to clean, order, and merge data frames in R. Graphs were generated using the package ggplot and its associated package gridextra. Statistical tests used include t-tests, analysis of variance and covariance (ANOVA and ANCOVA), Pearson's correlation, and Tukey HSD test (from the base R package). Both general linear models (glm) and mix models (from package lmer and lme4) were used to assess the relationship between various experimental parameters. The package Segmented was used in experiment 1 for breakpoint analysis. A specialized package, Plantecophys, was used to fit A/C_i curves and obtain the values of J_{\max} and V_{cmax} . Further the package emmeans was used to calculate the regression slopes and mean values of groups of data.

Experiment 1

Aims:

- 1) Assess genetic variation in WUE_{wp} amongst the target varieties based on their biomass accumulation per water use across early vegetative stages when exposed to soil drying
- 2) Assess the effect of genotype and soil moisture content on WUE_i as determined by A and g_s
- 3) Establish categorization of target varieties based on WUE_{wp} and WUE_i , to be used and refined in subsequent experimentation

Hypothesis:

- Landraces will perform similarly to cultivars for WUE_{wp} , but differ in their leaf level and mechanistic response
- Stomatal sensitivity to drying soil will vary between genotypes

Plant Material:

The first experiment served as an initial panel to determine how the traits of interest vary in these genotypes, thus all genotypes were used as specified in table 1.

Design:

Plants were grown under optimal watering conditions for 23 days from sowing to third leaf stage (3L), when one third of replicates (36) were used to determine initial plant fresh weight, plant dry weight, leaf water potential, and stomatal conductance.

Remaining plants were exposed to well-watered (WW, 36 plants) and water deficit (WD, 36 plants) conditions. WW plants received a saturating amount of water as needed to maintain above 70% of drained capacity. WD plants were re-watered to 60 % of drained capacity, after reaching at an estimated 20 to 30 % of drained capacity. Weight at a given proportion of drain capacity was determined as detail above. Irrigation amount for the WD treatment was determined by subtracting the pot weight from the estimated weight at 60% drained capacity (Appendix A - Table A.1). Upon implementing the watering treatments, pots were covered with black tape to minimize evaporative soil water loss. Three re-watering cycles that lasted 5 to 7 days were implemented while stomatal conductance and water use were measured daily. Dry down time was similar within genotypes but differed between genotypes, thus genotypes were re-watered watered on different days. Plants were consistently measured in the same pattern according to the EDGAR randomization, and watered following measurement of all replicates. WW and WD plants were harvested after 21 to 24 days of treatment, corresponding to three dry and re-wet cycles. Well-watered plants were harvested above 70% and water deficit below 30% of drained capacity respectively.

The day before intended harvest when water deficit was most extreme, LICOR sample measurements were conducted as described above. The day of harvest stomatal

conductance and pot weight were measured a final time, and samples were taken for ABA content. Subsequently, measurements for biomass, partitioning, leaf area, and GWC were conducted as outlined above.

Experiment 2

Aims:

- 1) Investigate impact of genotypic variation on whole plant and leaf level measure, such as partitioning and C_i , on expression of WUE_{wp} , WUE_i , and their associate parameters
- 2) Examine variation in foliar ABA content and sensitivity to clarify its role in regulating g_s and thus WUE_i and WUE_{wp}
- 3) Assess level of variation in the re-watering response of A and g_s by quantifying recovery post-irrigation

Hypothesis:

- Leaf area and leaf partitioning vary between genotypes and are correlated to WUE_{wp}
- Variation in WUE_i is linked to differences in stomatal sensitivity to soil drying associated with the level of ABA production under water deficit
- Rebound of A and g_s upon re-watering varies between genotypes

Plant Material:

Genotypes of interest were chosen based on the findings of experiment 1 (Table 4). GA and KR were selected as low and high WUE_{wp} varieties respectively. GA and KR also showed contrasting leaf partitioning. G1, G57, and G91, were all used as moderate WUE_{wp} and leaf partitioning varieties, that captured variation in stomatal sensitivity to soil drying (low, high, and moderate respectively).

Design:

Genotypes were selected as based on their stomatal sensitivity, WUE_{wp} , and leaf partitions as detailed above. The AP4 porometer was not used to measure stomatal conductance due to evident environmental sensitivity. The LI-6400xt was used to measure A , g_s , and WUE_i at multiple time points as detailed below.

Plants were well-watered until third leaf stage (25 days post sowing), when 4 replicates of each genotype were used for LICOR measurements, biochemical sampling, initial biomass (fresh and dry), initial leaf area, biomass partitioning, and GWC.

Following the initial harvest pots were relocated to Lancaster University's high throughput phenotyping platform (Ryan et.al. 2016, GH 14), re-randomized and covered with black tape to minimize evaporation. Two watering regiments were deployed in which half of the plants (40, 8 per genotype) received well-watered (WW) conditions and half (40, 8 per genotype) received water deficit (WD) conditions. WW plants received

optimal amounts of water, maintaining over 70% of DC. WD plants were permitted to dry to approximately 20% to 30% of drained capacity, at which point they were re-watered to an estimated 60% of drain capacity. Weight at a given proportion of drain capacity was determined as detail above. The watering cycle was repeated 3 times and lasted 5 to 7 days, resulting in 3 “dry” measurements and 3 “re-watered” measurements. Time to reach 20% drain capacity was similar within genotypes but differed between genotypes. Therefore, each genotype was measured when proportion of drain capacity was below near 20% (+/- 5%).

Throughout the experiment, the platform logged changes in pot weight which was used to estimate water use over the duration of the treatment. However, loss of 8.6 % of total water use data for all replicates occurred due to platform failure. LICOR sample measurements were taken before watering on the “dry” days near 20% of drain capacity then again on the subsequent “re-watered” days. Watering took place in the evening after “dry” measurements were completed. Such a set up allowed for the recovery of photosynthesis to be quantified..

Harvest was performed across two days, where half of the plants were harvested on the third “dry” day and half on the third “re-watered” day, in order to determine their phytohormonal response under both conditions. WD plants were harvested at a proportion of drain capacity that fell below 30% and as close to 20% as possible, while WW plants were harvested above 70% of drain capacity. Replicates were used for biochemical sampling, and measures of total biomass (fresh and dry), total leaf area, biomass partitioning, and GWC.

Experiment 3 and 4:

Aims:

- 1) Determine impact of leaf age on variation in WUE_i , it's parameters g_s and A
- 2) Investigate the role that ABA and ACC content and sensitivity plays in regulating g_s across leaf age
- 3) Examine impact of low water availability on achievable photosynthetic rate, to understand the contribution of stomatal and non-stomatal limitation to water deficit response (Experiment 3 only)

Hypothesis:

- WUE_i declines with leaf age as a result of changes in both g_s (due to ABA/ACC ratio) and A (due to decline in Rubisco activity)
- Cumulative WUE_i across leaf age will vary between genotypes
- Non-stomatal limitation to photosynthesis will vary between genotypes

Plant Material:

GA, KR, and G1 were used in both experiments due to their contrast in WUE_{wp} . G57 and G91 were dropped as they performed similar to G1, and fewer genotypes allowed

increased replication. GA again served as the low WUE_{wp} variety, and KR as the high WUE_{wp} variety. G1 also tended to show high WUE_{wp} and showed high levels of foliar ABA content under soil drying when compared to the two cultivars. In experiment 3, G83 was included as delayed data analysis indicated that it was of interest due to its high carbon assimilation (A). However, G83 was dropped in experiment 4 as it performed similar to G1 for virtually all measures.

Design:

The third and fourth experiments aimed to quantify WUE_i across leaf age, and were nearly identical with some exception. Experiment 3 was conducted in the glasshouse and examined four genotypes at three leaf ages. Alternatively, experiment 4 was conducted in a controlled environment room, and examined three genotypes at three leaf ages. The controlled environment in experiment 4 served to minimize measurement inaccuracy due to variable VPD and temperature as experienced in the glasshouse in experiment 3. Replicates in experiment 3 were also used to fit A/C_i curves under water deficit and well-watered conditions.

For assessing WUE_i across leaf age in both experiments, all plants were maintained at WW conditions. At fifth leaf stage (three weeks post sowing) measurements began, where the fourth leaf of each plant underwent sample LICOR measurements at three ages: partial leaf emergence (week 1, fifth leaf stage), full emergence (week 2), and onset of leaf aging (week 3). Plants were weighed post measurement and assumed to be at a soil moisture corresponding to that pot weight. The number of replicates measured per genotype in experiment 3 decreased as fifth leaves were sampled to 12 and then 6 on weeks 2 and 3 respectively. As LICOR stabilization is prolonged in the CE room, only six replicates from each genotype (total 18) could be measured weekly in experiment 4. Such a design ensures that each leaf sample corresponded to a LICOR sample measurement. On each measurement day, a subset of fifth leaves from each genotype was sampled following the protocol outlined in the above. These replicates were randomly selected using the EDGAR tool and assigning each individual a “sampling age” (i.e. week 1,2,3). Following sampling plants were retained for future A/C_i (experiment 3) and/or biomass measurements (Experiment 3 and 4) but were no longer subjected to sample LICOR measurements. On sampling days relative water content was also measured (see above).

The second component of experiment 3 served to understand the impact of water deficit on achievable net photosynthetic rate via A/C_i curves. Prior to the onset of the second component, half of the replicates (reps 1, 3, 5) were harvested for initial biomass. Remaining replicates (reps 2,4,6) were exposed to two watering regimens where one half received optimal watering (above 70% of drain capacity), and the other had water withheld until soil moisture reached an estimate of 20% of drain capacity. As water use was high at this stage, risk of drain capacity falling below 20% was prevalent. To mitigate this risk WD plants were retained at suboptimal soil moisture by watering to approximately 50% of drain capacity every evening. By measurement on the following day, soil moisture had returned to 20% of drain capacity. Water use was determined daily.

When fitting A/C_i curves on water deficit plants, stomatal closure makes it difficult to accurately determine levels of C_i and A . To account for stomatal closure in water deficit replicates clear autoclave bags were placed over plants for at least 30 minutes to increase local humidity and encourage stomatal opening. A/C_i curves were then produced as described above. Six replicates from each genotype were measured for A/C_i curves, with three from each treatment. Measurements took place at full flag leaf emergence, pre-anthesis. Genotypes were measured in groups due to differences in development rate, where KR and G83 were measured in one group across 2 days and G1 in another across 2 days (approximately 6 plants per day). GA was never measured as no flag leaf emerged.

After A/C_i (experiment 3) and leaf age (experiment 4) measurements were complete for all genotypes, destructive measurements were taken for above ground biomass and gravimetric water content.

Results:

Experiment 1

All genotypes showed a linear relationship between water use (WU) and biomass gain (BM), regardless of the irrigation treatment suggesting proportional changes in these variables under water deficit (Figure 4_a, Appendix C - Table C.1). Further, no genotypic variation was observed for BM or WU, but water deficit significantly decreased both variables in all genotypes. The slope of BM *versus* WU represents WUE_{wp} (Figure 4_a, b), of which there was nearly a two-fold difference between the highest and lowest performing genotypes (G83 and GA, respectively). G1 performed at a median level and showed no significant deviance from any other genotypes. G83 performed similarly to the drought tolerant and high WUE_{wp} cultivars DR and KR, indicating potential for high tolerance to water deficit.

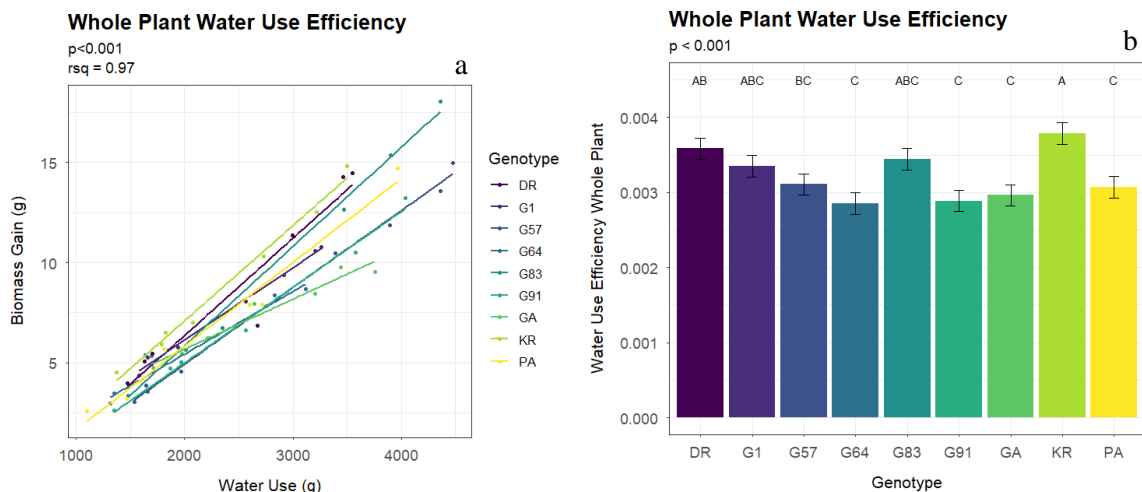


Figure 4: a) Lines represent the rate of biomass gain per water consumed over the treatment period, where each point represents an individual plant b) Bars represent the slope of the relationship between biomass gain and water consumed (WUE_{wp}) in figure 4_a +/- standard error. Letters indicate significance groupings via.

Leaf area (LA) varied between treatment and genotype with a significant interaction between the two (Appendix B – Table B.1). Under WD conditions, genotypes showed no variation in leaf area however, under WW conditions leaf area of G57 was 2.3x higher than G64. G57 and G1 had the largest and smallest decreases in LA due to water deficit, respectively. Greater LA was correlated to greater WU, BM and leaf biomass (LB) (Appendix C – Table C.1) and G57 exhibited the greatest rate of increase in LA per unit BM when compared to the lowest ranking genotypes (G83 and KR). A lower rate of increase in LA per unit BM was associated with genotypes showing high WUE_{wp} (e.g. G83 and DR). Regardless of genotype, higher LA led to greater water loss, but increased leaf biomass.

Leaf biomass (LB) also varied between treatment and genotype, where genotypes only differed under WW conditions. Leaf biomass of G57 was 2.2x greater than that of DR regardless of treatment, and G57 and G64 showed the largest and smallest decline in LB under water deficit respectively. The relationship between LB and WU varied between genotypes indicating differential increase in WU per unit gain in LB (Figure 5a). Further, mean leaf biomass partitioning ($LP = LB$ per BM) differed between treatment and genotype (Appendix B- Table B.1). However, no interaction was observed between treatment and genotype, thus WD genotypes showed no deviation from their WW counterparts. Regardless of treatment, a two-fold difference in LP was observed between GA (highest) and KR (lowest). Increased LP was significantly correlated to decreased WUE_{wp} across all genotypes (Appendix C - Table C.1, Figure 5b), as higher LB increased WU as a result of more transpiring tissue.

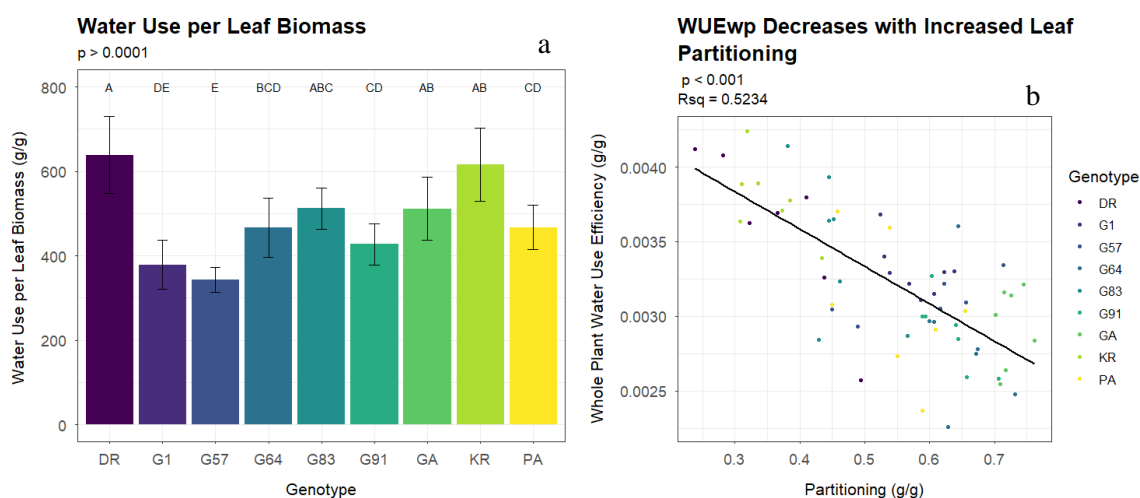


Figure 5: a) Bars represent the slope of the relationship between water use and leaf biomass gain over the treatment period +/- the standard error b) the line represents relationship between WUE_{wp} and final leaf partitioning where each point represents an individual plant.

WUE_i was approximately doubled under water deficit but showed no genotypic variation. Increased assimilation (A) was significantly correlated to increased stomatal conductance (g_s) (Appendix C – Table C.1), and water deficit decreased A and g_s by 32 % and 71 % respectively. Since A declined at an increased rate as stomata closed (Curvilinear

relationship), the decline in g_s under water deficit was largely responsible for the increased WUE_i .

Stomatal sensitivity to soil drying differed between genotypes as indicated by variability in the rate of stomatal closure as gravimetric water content (GWC) decreased (Figure 6a). Variation was only observed after a soil moisture threshold (“break-point”), which was similar between genotypes (Figure 6b). Stomatal conductance of GA was more sensitive to soil drying (1.8-fold) than G64 which was the least sensitive. DR and G57 were similar to GA for stomatal sensitivity to soil drying, and G83 and G1 were similar to G64 (Figure 6a). Genotypes with high WUE_{wp} experienced both high (DR) and low (G83) sensitivity to soil drying. Thus, genotypes vary for stomatal decline under water deficit which interacts with other whole plant traits to achieve the whole plant phenotype.

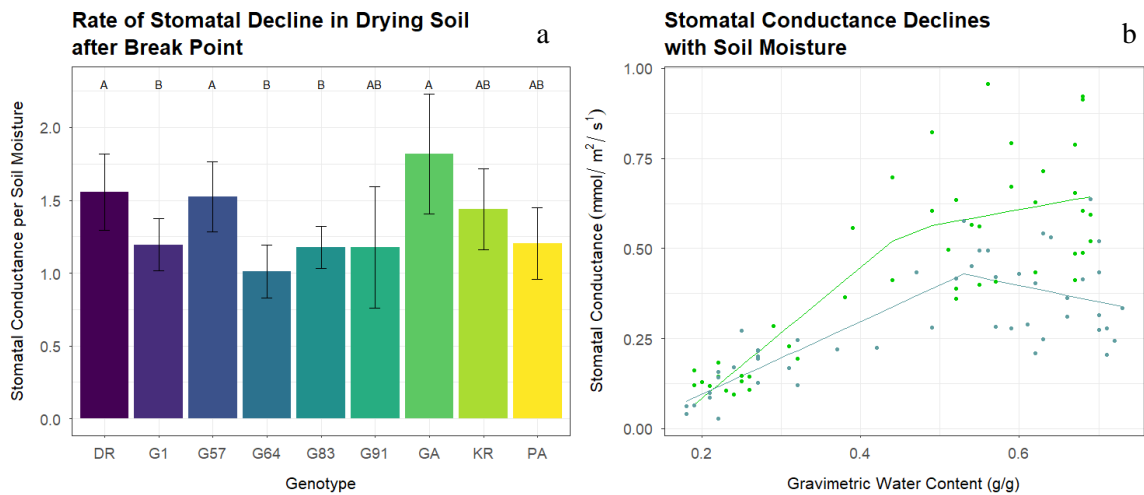


Figure 6: a) Bars represent the slope of g_s to GWC \pm the standard error, where higher level indicates greater stomatal sensitivity to soil drying, letters indicate significance groupings determined b) Lines represent the relationship between stomatal conductance and gravimetric water content where each point represents a single measurement point on each of the 4 replicates. The green line represents the most sensitive genotype GA, and the blue line represents the least sensitive genotype G64.

Foliar ABA content ($[ABA]_f$) did not vary between treatment and genotype when analyzed in four genotypes of interest (GA, G1, G83, and KR). Nevertheless, $[ABA]_f$ increased with soil drying (Appendix C – Table C.1, Figure 7a) and stomatal conductance declined as $[ABA]_f$ increased (Appendix C – Table C.1, Figure 7b), however these curvilinear relationships showed no variation between treatment or genotype indicating no differences in foliar accumulation of ABA or stomatal sensitivity to $[ABA]_f$.

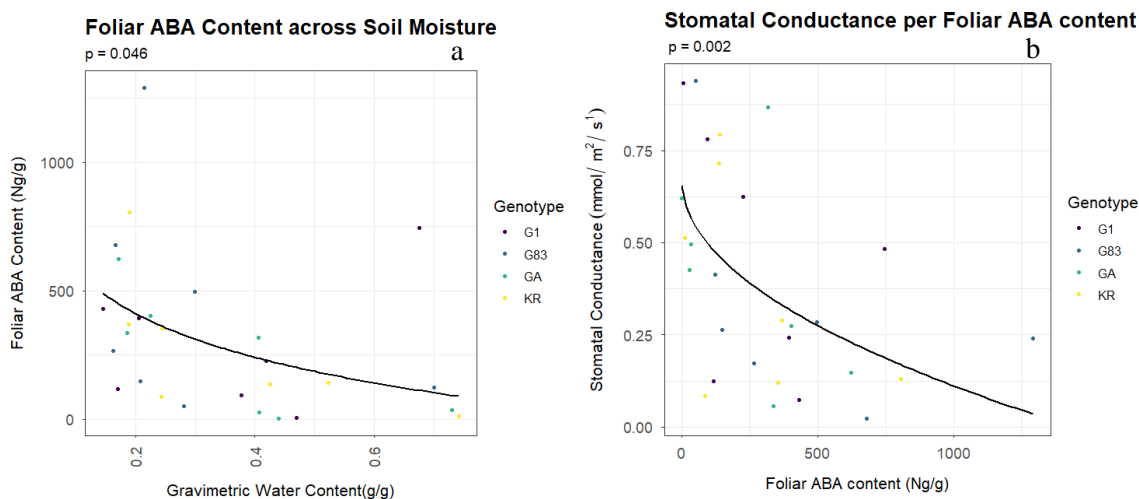


Figure 7: a) the curvilinear line represents the relationship between foliar ABA content and Gravimetric Water Content at harvest, where each point represents an individual plant b) The curvilinear line represents the relationship between stomatal Conductance and foliar ABA content at harvest, where each point represents an individual plant

WUE_i and WUE_{wp} were not correlated, while the parameters used to calculate these measures were. Increased WU correlated to higher g_s , and increased BM to higher A (Appendix C, Table C.1). Thus, variation in the mechanistic regulation of WU by g_s and of BM by A was suspected to exist.

From the results in experiment 1, genotypes were ranked based on the WUE_{wp} , leaf partitioning, and stomatal sensitivity to soil drying to select contrasting genotypes for subsequent experiments (Table 4).

Key take-aways:

- Genotypes show significant variation for WUE_{wp}
- Stomatal sensitivity to soil drying varies between genotypes, but does not correlate to WUE_{wp}
- Increased leaf partitioning correlates to lower WUE_{wp}
- Genotypes G1, G57, G91, GA, and KR capture the variation observed for these traits and were used in subsequent experimentation

<i>Genotypic rankings for three traits of interest</i>				
<i>Genotype</i>	WUE _{wp} grouping	<i>g_s</i> grouping	LP grouping	Rank
G1	Moderate	Low	Moderate	7
G57	Moderate	High	Moderate	5
G64	Low	Low	Moderate-high	9
G83	High	Low	Low	3
G91	Moderate	Moderate	Moderate-high	6
DR	High	High	Low	1
GA	Low	High	High	8
KR	High	Moderate	Low	2
PA	High	Moderate	Moderate-low	4

Table 4: Rankings of genotypes from experiment 1 based on WUE_{wp} (high – high WUE_{wp}), *g_s* (high = high stomatal sensitivity), and LP (high = high leaf biomass per total biomass). High WUE_{wp} was the primary rank indicator, followed by Low LP and then high *g_s*. Bolded genotypes were used in subsequent experimentation.

Experiment 2

The second experiment aimed to determine repeatability in WUE_{wp}, examine WUE_i at multiple time points, and establish the effect of re-watering on assimilation rebound. Five (GA, G1, G57, G91, and KR) were selected based on their contrasting performance in Experiment 1 (Table 4).

Again, BM had a linear response to WU regardless of treatment (Figure 8a, Appendix C – Table C.2). Further, WU and BM varied between treatment and genotype (Appendix B, Table B.2, data not shown). Genotypes differed for WU and BM under WW conditions, but not WD conditions. In WW conditions G57 showed 1.8x greater BM than GA, and 1.5x greater WU than KR. Thus, water deficit restricted BM and WU the most in G57, while KR and GA showed the lowest declines in BM and WU respectively. WUE_{wp} (the slope of the relationship between WU and BM) was 2.2-fold higher in KR than GA, however all other genotypes performed similarly to KR (Figure 8b).

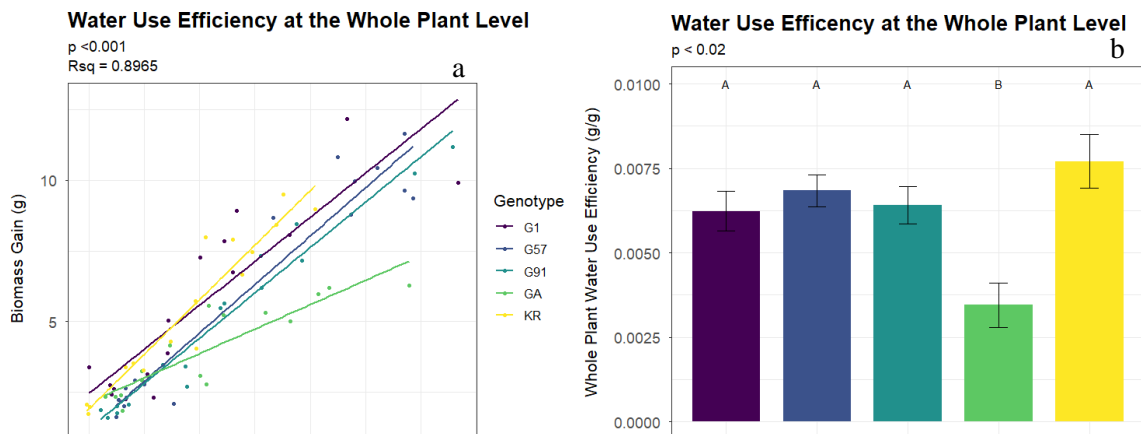


Figure 8: a) The line represents the relationship between biomass gain and total water use over the treatment period, each point represents an individual plant and are coloured according to genotype b) Bars represent the slopes depicted in figure 8a +/- the standard error and indicate the rate of change in biomass per unit of water consumed (WUE_{wp}) over the treatment period, letters indicate significance groupings.

Leaf area, leaf biomass, and leaf partitioning differed between treatment, genotype, and their interaction (Appendix B, Table B.2) where genotypes differed under WW conditions but not WD. Under WW conditions LA and LB of G57 were 3.4 x and 2.9x higher than KR, which displayed the lowest LA and LB. Reduction of LA and LB under water deficit was the highest in G57, and the lowest in GA (LA) and KR (LB). Further, GA and KR displayed the highest and lowest LP respectively regardless of treatment. However, water deficit increased LP in KR, G1, and G57 ($p < 0.04$). Additionally, increased LB and LA were strongly correlated to increased BM and WU but weakly correlated to increased WUE_{wp} (Appendix C – Table C.2). The relationship of both LA and LB to BM varied between genotypes where KR saw the greatest gains in BM per unit LB or LA, and GA the least. Thus, increased leaf tissue results in greater water loss but also biomass gain.

Genotypes did not vary for WUE_i as determined by the relationship of A to g_s (Figure 9_{a,b}, Appendix C – Table C.2). Water deficit elicited a 7 % increase in WUE_i across all genotypes ($p = 0.0374$). Further, A and g_s varied between treatment and genotype, with WD decreasing A and g_s by 46% and 47% respectively. KR had the highest A and g_s under both treatments, which were 1.3x and 1.6x higher than the A and g_s values of G57 regardless of treatment. G57 performed similarly to all remaining genotypes which also differed from KR. Additionally, treatment, and not genotype, negatively impacted the relationship of A and g_s to GWC as assimilation and stomatal conductance were lower in WD versus WW plants at a given soil moisture. The lack of genotypic variation in stomatal sensitivity to soil drying may be attributed to a narrow range of soil moisture and lower measurement replication. Regardless, decline of A followed that of g_s resulting in a similar rate of decline across soil drying.

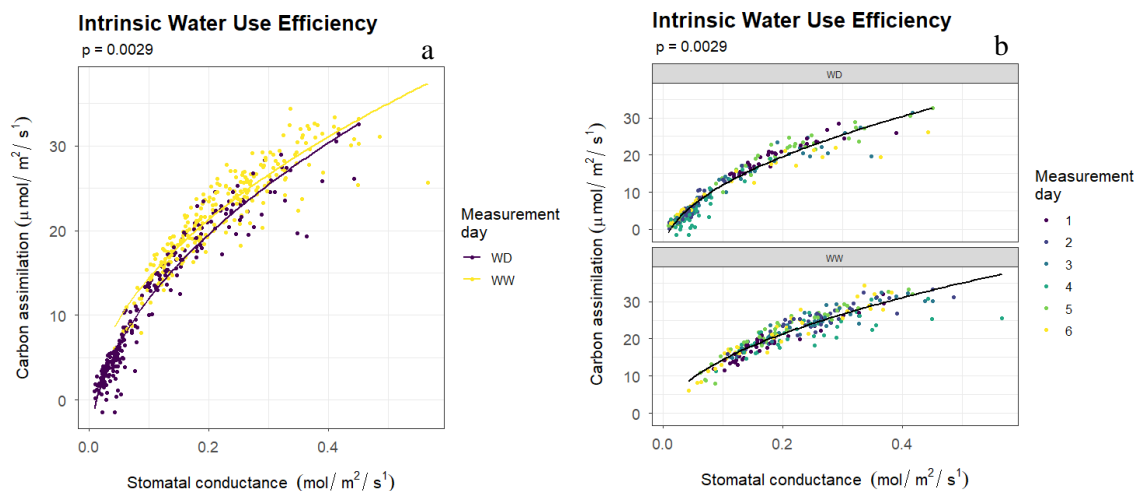


Figure 9: a) The curvilinear line represents the relationship between carbon assimilation (A) and stomatal conductance (g_s), each point represents an individual measurement where each plant was measured at multiple time points. Lines are coloured by watering regimen where WW = well-watered and WD = water deficit b) lines represent the relationship between carbon assimilation (A) and stomatal conductance (g_s), points are coloured by measurement day where days 1, 3, and 5 were “re-watered” days, days 2 and 4 were “dry” days and day 6 was the final harvest. The top panel depicts those replicates under water deficit (WD) conditions, and the bottom panel depicts those under well-watered (WW) conditions.

Foliar ABA content ($[ABA]_f$) increased as soil moisture decreased, via a curvilinear relationship that varied across genotypes (Figure 10a, Appendix C -Table C.2). G1 showed a 5.2-fold higher increase in $[ABA]_f$ as the soil dried compared to GA, however both genotypes were similar to all others. Stomatal conductance declined as $[ABA]_f$ increased, similarly in all genotypes again indicating no differences in stomatal sensitivity to $[ABA]_f$ (Figure 10b, Appendix C -Table C.2).

Upon re-watering, genotypes did not show significant or consistent recovery of assimilation. Although, the level of A per C_i (“Assimilation efficiency”) declined as stomata closed where the rate of decline was 35% higher in WD plants and was unimpacted by genotype. Such evidence indicates potential non-stomatal limitation to photosynthesis across all genotypes.

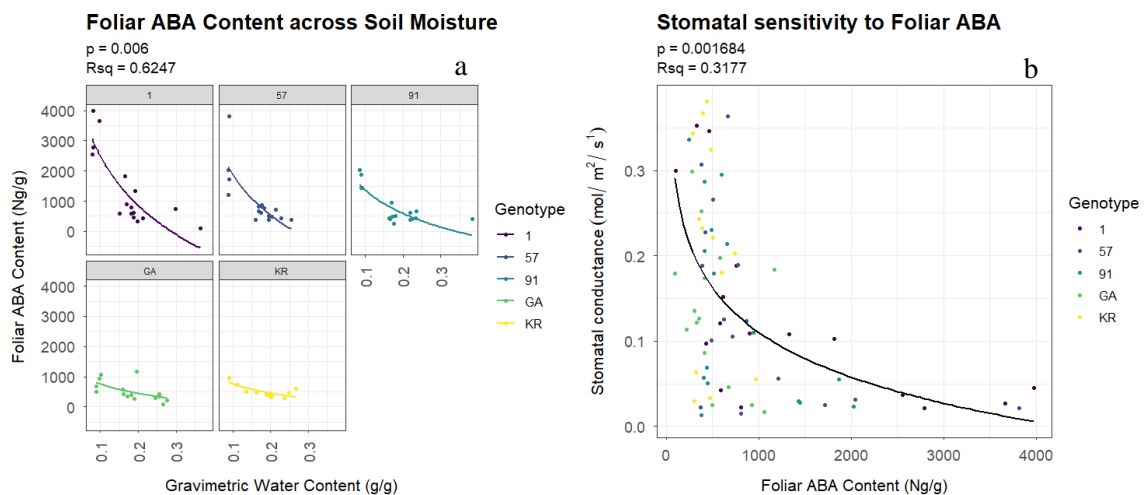


Figure 10: a) The curvilinear lines represent the relationship between foliar ABA content $[ABA]_f$ and gravimetric water content, each point represents an individual sample collected from each plant at harvest b) The curvilinear lines represent the relationship between stomatal conductance and foliar ABA content $[ABA]_f$, each point represents an individual sample collected from each plant at harvest

WUE_i and WUE_{wp} showed a weak positive correlation that was unimpacted by genotype (data not shown, Appendix C – Table C.2).

Key take-aways:

- Variation in WUE_{wp} is consistent and stable
- Water deficit results in an increase in WUE_i in wheat
- Increased leaf biomass and leaf partitioning corresponds to an increase in water use
- Genotypes vary for increase in foliar ABA content under soil drying, but not stomatal sensitivity to ABA
- Genotypes GA, KR, and G1 show the most contrasting responses for WUE_{wp} , leaf partitioning, and foliar ABA content under soil drying and should be used in subsequent experiments.

Experiment 3

The objective of experiment 3 was to determine the impact of leaf age on WUE_i , g_s , A , and their relationship to the whole plant phenotype. Additionally, limitation of A under WD was examined by fitting A/C_i response curves.

WUE_{wp} (the slope of the relationship between WU and BM) did not vary between treatment or genotype (Figure 11a, Appendix B – Table B.3). However, BM at a given WU varied between genotypes as indicated by the mean WUE_{wp} (Figure 11b, Appendix B – Table B.3). Mean WUE_{wp} of G83 was 2.5-fold greater than the lowest ranking genotype, GA. Treatment, genotype, and their interaction significantly affected BM and WU (Appendix B – Table B.3). Water deficit decreased both WU and BM, with no genotypic variation under WD. Under WW conditions, G83 produced 2.5x more biomass than GA, while G1 consumed 1.6x more water than KR. G1 and KR showed the largest (54%) and smallest (28 %) decline in water use under water deficit respectively. Water deficit had no impact on BM of KR and GA, but decreased BM of G1 and G83 by 55% and 50% respectively. Thus, genotypes vary for their ability to restrict biomass gain or water use under water deficit, resulting in variation in WUE_{wp} .

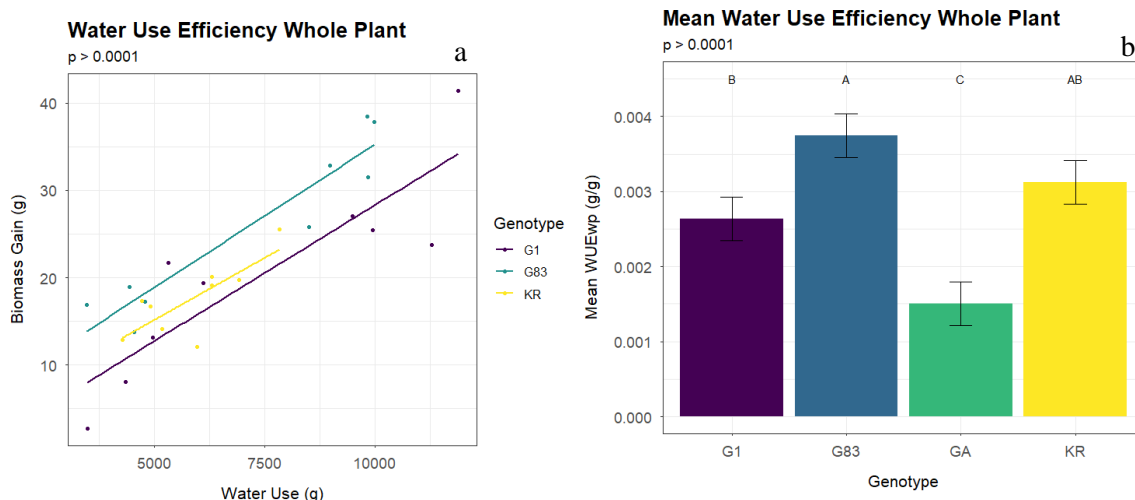


Figure 11: a) Lines represent the relationship between biomass gain and water use across the treatment period, where each point represents an individual plant and are coloured by genotype b) Bars represent the mean WUE_{wp} (biomass gain/water use) across genotypes +/- the standard error, which approximates the position of the line in figure 11a, letters indicate significance groupings determined via Tukey method

WUE_i varied significantly between genotypes as determined by the slope of the relationship between A and g_s and the mean WUE_i across leaf age (Appendix B – Table B.3). Mean WUE_i of KR was 28% and 33% lower than that of GA and G1. Leaf age did not affect the response of A to g_s , or the mean WUE_i of genotypes across replicates. The relationship between A and g_s was positive and curvilinear (Figure 12a, Appendix C – Table C.3) and both A and g_s varied between genotype and leaf age (Figure 12b,c, Appendix B, Table B.3). KR displayed 24% higher A and 67% higher g_s than G1, resulting in lower WUE_i . Leaf ageing decreased both A and g_s regardless of genotype, with genotypic variation between leaf age for A but not g_s . KR and G83 maintained A as

leaves aged, however A significantly decreased by 24% and 31% from week one to three in G1 and GA respectively. Ability to maintain A across leaf age was associated with high WUE_{wp} genotypes (KR and G83).

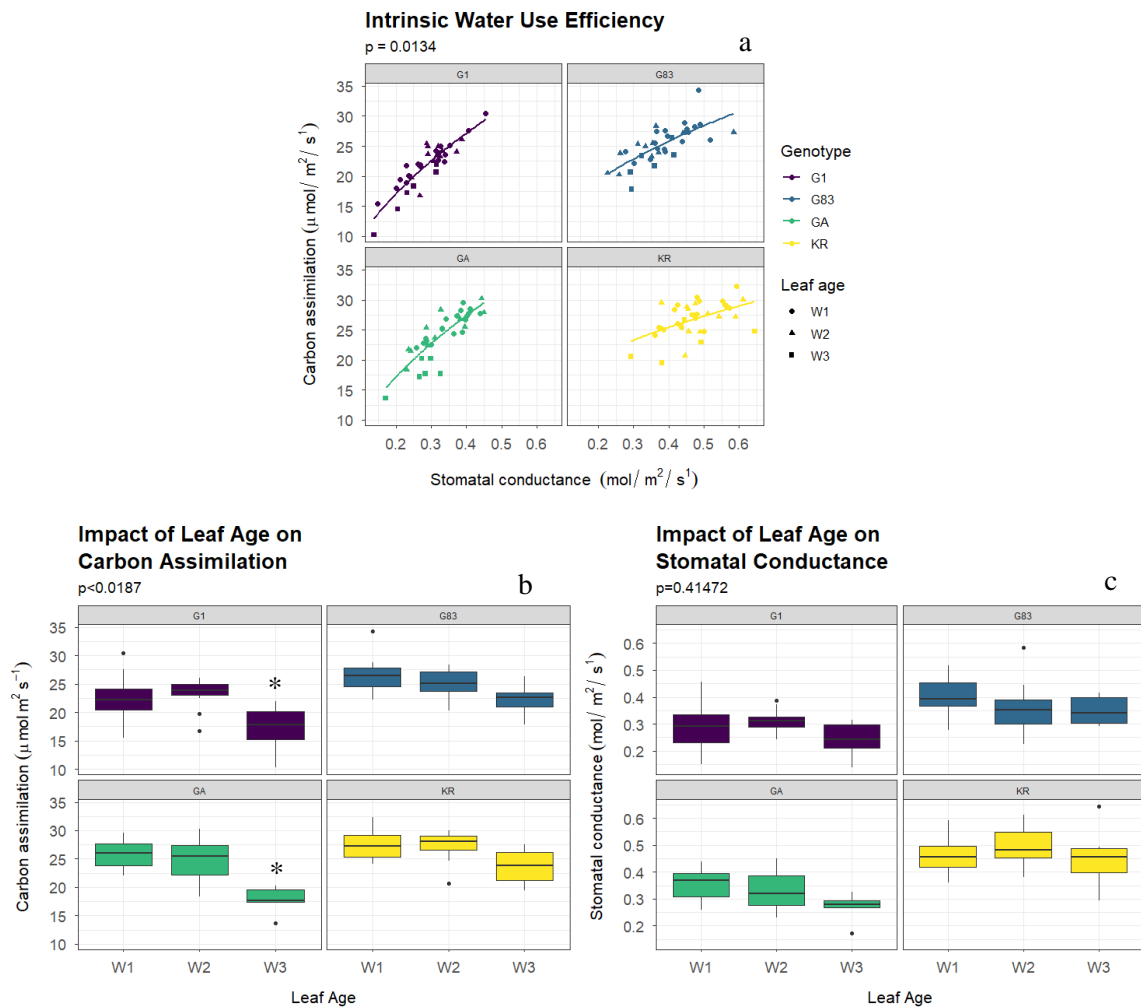


Figure 12 a): Lines represent the curvilinear the relationship of carbon assimilation (A) to stomatal conductance (g_s), each point represents an individual measurement for various replicates across three measurement dates b) the box plot represents the impact of leaf age on carbon assimilation where the * designates a significant decline in A compared to week 1 c) the box plot represents the impact of leaf age on carbon stomatal conductance. In all panels Week 1 = W1, Week 2 = W2, and Week 3 = W3

Both A and g_s were negatively correlated to WUE_i , although the correlation was weaker between A and WUE_i than g_s and WUE_i (Appendix C – Table C.3). The relationship between g_s and WUE_i varied between leaf age and not genotype (Figure 13), where decline in WUE_i per unit increase of g_s in week three was approximately 30 % lower than that of preceding weeks. Genotype and leaf age did not affect the relationship of A to WUE_i . Furthermore, genotype and leaf age did not affect the negative correlation of WUE_i to C_i (Figure 14) or the positive correlation of A and g_s to C_i (Appendix C – Table C.3). Thus, stomatal conductance directly affects WUE_i by regulation of C_i and thus A which both also influence WUE_i .

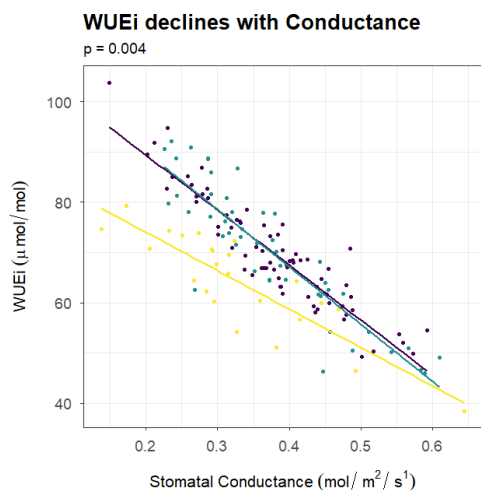


Figure 13: Lines represent the relationship between WUE_i and g_s at different leaf ages. Each point represents an individual measurement of a various genotypes and colours indicate the leaf age where W1 = week 1, W2 = week 2 and W3 = week 3.

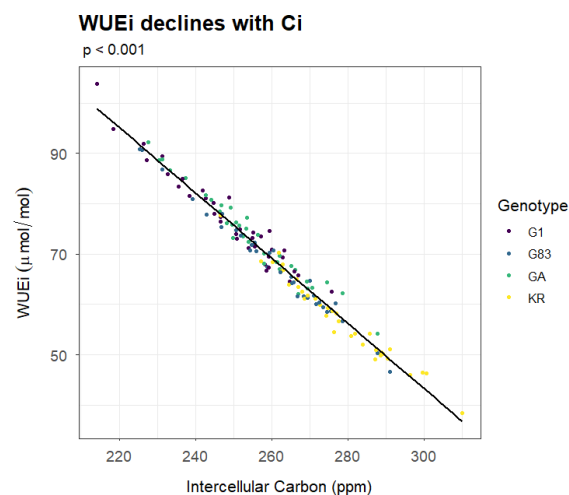


Figure 14: The line represents the relationship between WUE_i and C_i. Each point represents an individual measurement of a various genotypes at multiple leaf ages and colours indicate genotype

Foliar ABA ([ABA]_l) of these WW plants did not vary across genotype or leaf age (Appendix B – Table B.3). Additionally, [ABA]_l was not significantly correlated with either g_s or relative water content (RWC) (Appendix C- Table C.3), again indicating a lack of variation in stomatal sensitivity to soil drying

A/C_i curves indicate that V_{cmax} and J_{max} varied between both treatment and genotype (Appendix B – Table B.3), however no variation was noted between treatments within genotypes (figure 15_{a, b}). KR had the highest V_{cmax} and J_{max} which were 1.4x and 1.2x greater than G1, which displayed the lowest values (Figure 15_{a, b}). V_{cmax} and J_{max} were positively linearly correlated (Figure 15_d, Appendix C - Table C.3), but treatment and genotype did not affect the relationship. WUE_i and WUE_{wp}, again, showed no significant correlation (Appendix C, Table C.3)

Key take-aways:

- Genotypes vary for their ability to sustain A as leaves age
- A declines before g_s as leaves age
- WUE_i varies between genotypes when measured across leaf age
- Foliar ABA content does not vary in well-watered conditions or across leaf age
- Metabolic limitations to A vary between genotypes and correspond to WUE_{wp} and decline in A

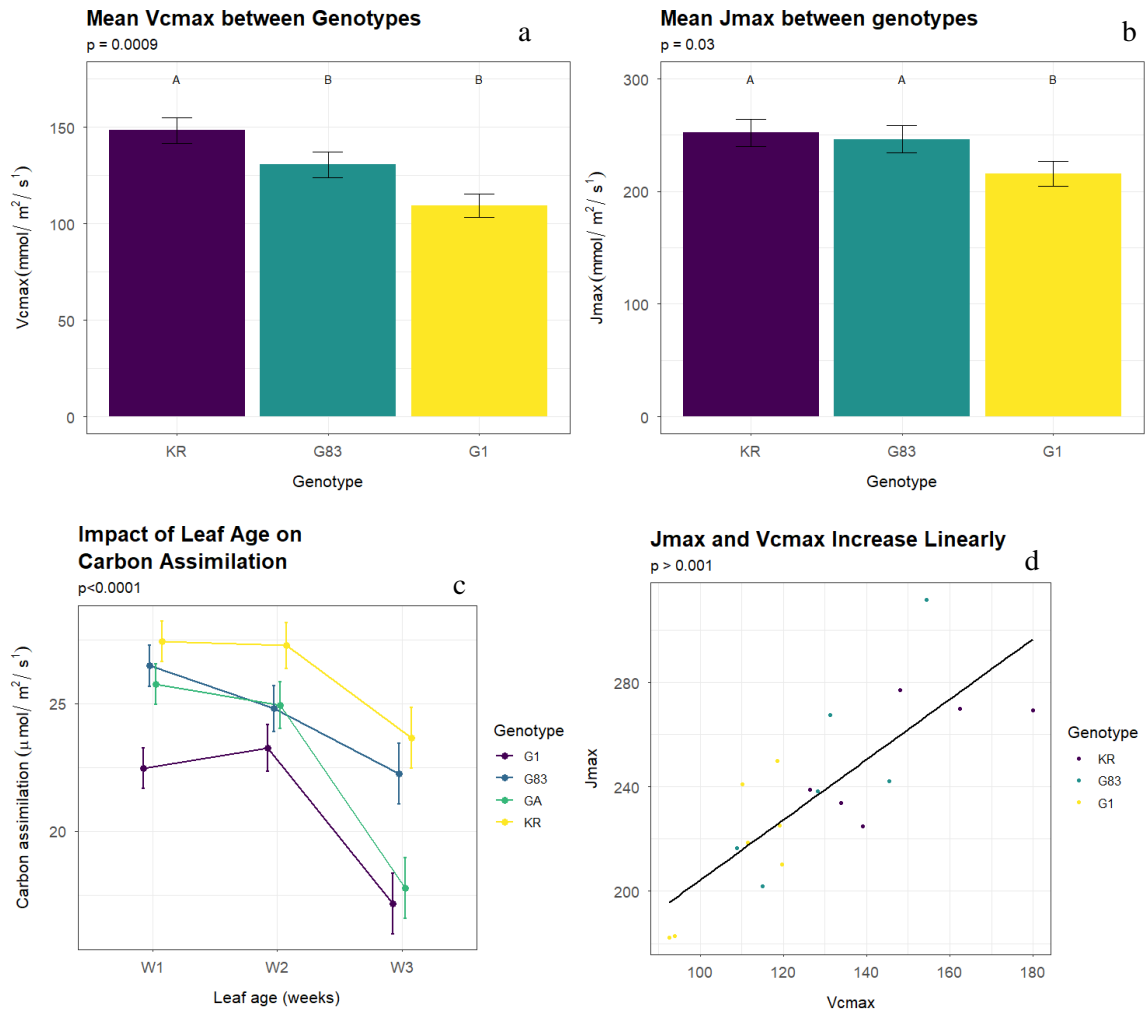


Figure 15: a) Bars represent the mean v_{cmax} between all replicates \pm the standard error as determined from fit A/C_i curves. V_{cmax} varied between genotype but not treatment. Letters indicate significance groupings, determined via Tukey method. b) Bars represent the mean J_{max} between all replicates \pm the standard error as determined from fit A/C_i curves. J_{max} varied between genotype but not treatment. Letters indicate significance groupings, determined via Tukey method. c) Lines indicate the mean decline in A , where each point is the mean A value across replicate. W1 = Week 1, W2 = Week 2, and W3 = Week 3. Lines

Experiment 4:

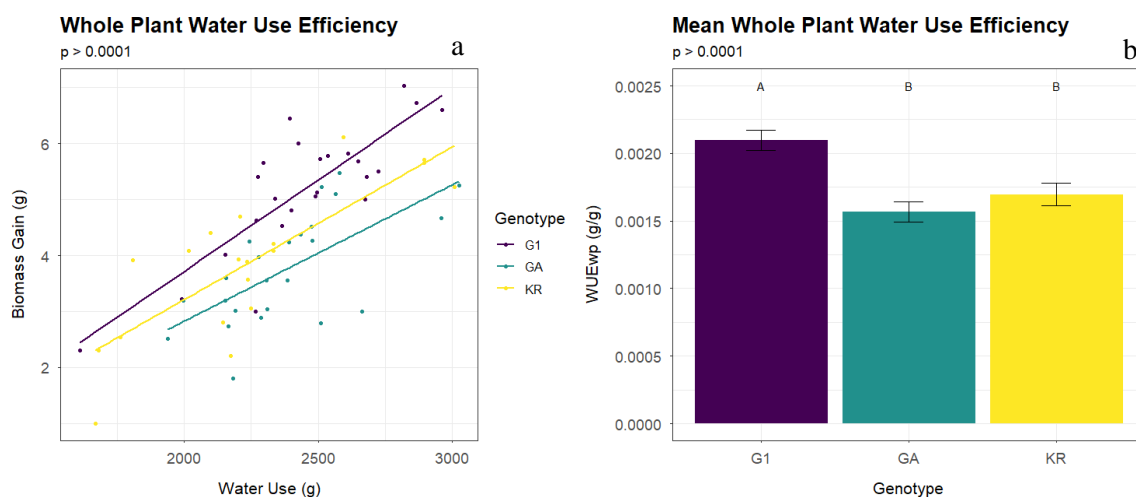


Figure 16: a) Lines represent the relationship between biomass gain and water use (WUE_{wp}), each point represents a single plant where colours indicate genotype b) Bars represent the mean WUE_{wp} (Biomass / Water use) between genotypes +/- the standard error and approximated the position of the line in Figure 16_a

Again, treatment and genotype did not affect WUE_{wp} (the slope of the relationship between WU and BM - Figure 16_a, Appendix B – Table B.4). However, BM at a specified WU (mean WUE_{wp}) did vary, with mean WUE_{wp} of G1 1.3x and 1.2x higher than GA and KR, respectively (Figure 16_b). Although G1 produced significantly more biomass than GA and KR, there was no genotypic variation in WU (Appendix B- Table B.4). Further, increased BM and WU were similarly correlated to increased WUE_{wp} across all genotypes (Appendix C – Table C.4). Biomass accounted for 86% of the variation in WUE_{wp} while water use only accounted for 44% of the variation (based off R^2 values). Such evidence indicated that WUE_{wp} is driven by BM under well-watered conditions.

Leaf partitioning (LP) and leaf biomass (LB) again showed genotypic variation, with GA and G1 displaying 1.6-fold greater LP and LB than KR respectively (Appendix B – Table B.4). Rate of increase in LP per unit LB varied between genotypes, where the rate was 1.5x greater in GA compared to KR, indicating differential contribution of leaf tissue to total biomass. Further, increased LB, but not LP, was significantly correlated to increased WU, BM, and WUE_{wp} (Appendix C – Table C.4). The relationship between LB and BM showed similar variation to that of LB and LP. Such relationships indicate that increased leaf biomass results in increased WU but also BM, leading to an increase in WUE_{wp} under well-watered conditions.

In these well-watered plants foliar ABA ($[ABA_I]$) content showed no significant variation between genotype or leaf age (Appendix B, Table B.4), and no significant relationship was observed between $[ABA_I]$ and g_s or relative water content (RWC) (Appendix C, Table C.4).

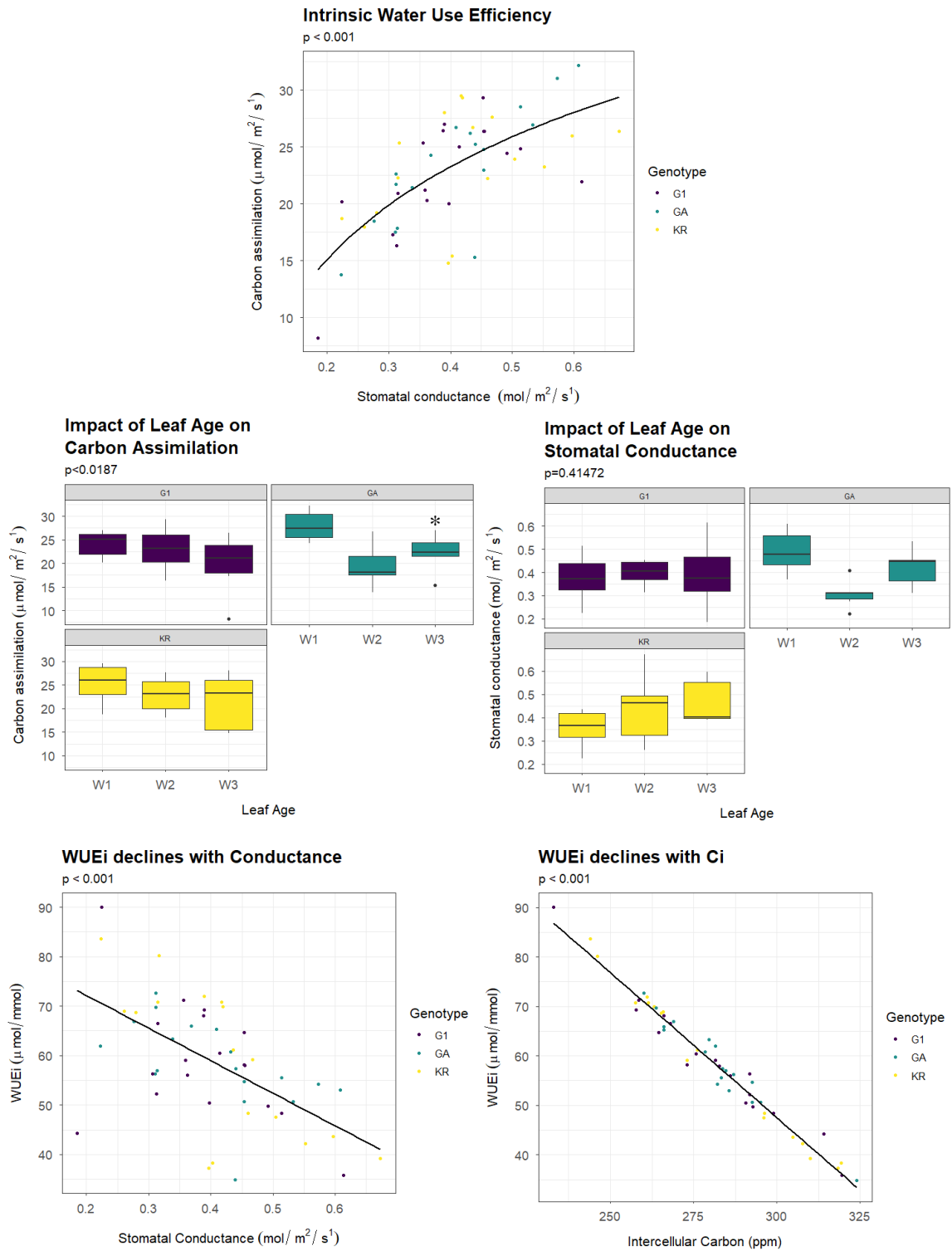


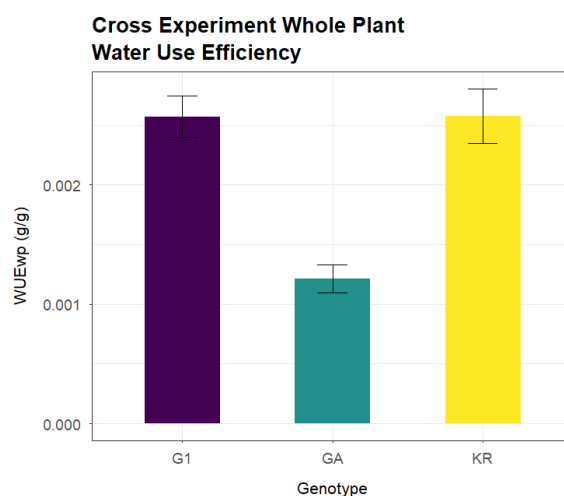
Figure 17: a) The curvilinear line represents the relationship between carbon assimilation (A) and stomatal conductance (g_s), each point represents an individual measurement for plants at multiple leaf ages b) the box plot represents the impact of leaf age on carbon assimilation where the * designates a significant decline in A compared to week 1 c) the box plot represents the impact of leaf age on carbon stomatal conductance. In all panels Week 1 = W1, Week 2 = W2, and Week 3 = W3 d) The line represents the relationship between WUE_i and g_s , each point represents a single plant and colours indicate genotype e) The line represents the relationship between WUE_i and C_i , each point represents a single plant and colours indicate genotype

No variation was observed in the relationship between A and g_s (i.e. WUE_i) across genotype or leaf age (Figure 17_a, Appendix B, Table B.4). Although g_s did not decline with leaf age, and A was maintained across leaf age in G1 and KR, A of GA decreased by 20% between weeks 1 and 3 (figure 17_{b,c}). Increased g_s was similarly correlated to decreased WUE_i across all genotypes (Appendix C, Table C.4), whereas A was not correlated with WUE_i . Higher intercellular CO_2 concentrations (C_i) correlated with decreased WUE_i and increased g_s and A (Figure 17_{d,e} Appendix C, Table C). Thus, WUE_i is highly regulated by changes in g_s unless other non-stomatal limitations exist and is not correlated to WUE_{wp} .

Key take-aways:

- Genotypes consistently vary for their ability to sustain A as leaves age
- A consistently declines before g_s as leaves age
- Stricter control of VPD and temperature results in no detectable variation in WUE_i

Cross Experiment Summary



b

Figure 18: Bars represent the slopes of BM *versus* water use in G1, GA, and KR across all 4 experiments +/- the standard error

The genotypes GA, KR, and G1 were consistently used across experiments due to their apparent differences in WUE_{wp} (Figure 18). They were subjected to a cross experiment analysis to determine if significant correlation of measurements existed. Each genotype showed significant positive correlation between BM, WU, and LB indicating that in all genotypes increases in biomass, and specifically leaf biomass, led to increased water use. Consistently, WU and WUE_{wp} were negatively correlated (Appendix C, Table C.5) while no clear correlation was evident between BM and WUE_{wp} .

WUE_{wp} and WUE_i showed significant positive correlation between all genotypes (Appendix C, Table C.5) where the value of WUE_i used was recorded on the sampling day of each individual. Therefore, as WUE_i increases, gains are observed at the whole

plant level. However, this relationship was only detectable when examined across variable growth conditions. The relationship was linear, where WUE_i accounted for 41.9, 56.7, and 40.4 % of increase in WUE_{wp} in KR, GA, and G1 respectively (Figure 19).

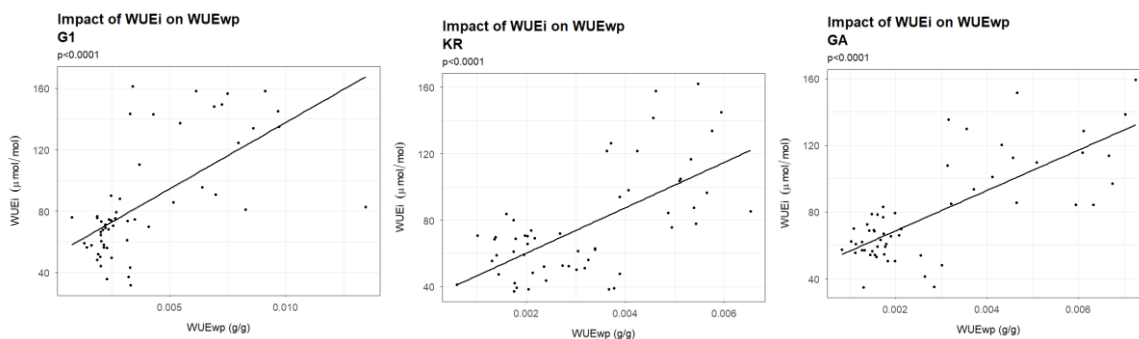


Figure 19: Lines depict the correlation between WUE_i and WUE_{wp} across all experiments for three genotypes of interest. From left to right, genotypes are G1, Gatsby, and Krichauff. Each point represents an individual plant from a single experiment. The WUE_i used is the value on the sampling day.

Additionally, correlations noted within experiments were also detected within genotypes across experiments. Specifically, WUE_{wp} was negatively correlated with both LP and C_i (Appendix C, Table C.5), where these relationships were significant without accounting for the interaction of other parameters. Further, g_s was positively correlated to C_i and A (Appendix C, Table C.5), suggesting significant regulation of carbon gain via stomata and thus their sensitivity to water deficit. Increased RWC was correlated with increased g_s in the genotypes GA and KR and with increased GWC in all genotypes (Appendix C, Table C.5). However, g_s tended to decrease ($p = 0.07248$, Appendix C – Table C.5) as the RWC declined in G1. Further, increased $[ABA]_l$ was correlated with decreased g_s and GWC in genotype G1 and KR but not GA (Appendix C - Table C.5). However, decreased g_s was correlated to decreased GWC in all genotypes. Genotypic variation in these relationship across experiments suggests variation in the mechanism regulating leaf water status under water deficit, as discussed below.

Key take-aways:

- WUE_{wp} shows similar genotypic variation across experiments, indicating stability across environments
- Leaf biomass partitioning is a strong indicator of WUE_{wp}
- WUE_i and WUE_{wp} show significant correlation when examined across multiple environments

Discussion:

Genotypic variation in WUE_{wp} and Whole Plant Measures

Across soil drying, the presented genotypes exhibited consistent variation in rate of biomass gained per water consumed (WUE_{wp}). However, variation was not observed in situations where no water deficit was administered, or treatment was administered at a late stage of development (experiments 3 and 4). It has previously been noted in wheat,

rice, and tomato that WUE_{wp} varies across development stages (Tatar, Brück, and Asch, 2016, Alou et.al. 2018, Li et.al. 2019). Therefore, it is probable the lack of variation observed in experiments 3 and 4 is the result of developmental differences and narrow range of water use respectively.

Variation in WUE_{wp} was consistently correlated to BM, while correlation to WU was always lower or insignificant. Biomass gain accounted for 40 to 86 % of variation across experiments regardless of genotype, suggesting that changes in WUE_{wp} are largely attributed to plant biomass. However, WU and not BM was consistently correlated to WUE_{wp} in the three genotypes of interest (GA, KR, and G1). Further, BM varied between genotypes across all experiments, indicating that it is likely the driving factor of variable WU and thus WUE_{wp} in wheat.

Leaf biomass and partitioning were also consistently correlated to WU, BM, and WUE_{wp} , where higher LB and LP had a negative effect on WUE_{wp} . Such a response is expected when considering that transpirational water loss occurs through the leaf, thus higher levels of leaf tissue result in increased water consumption. Leaf biomass and partitioning have been shown here and previously to be maintained across water deficit in wheat (Tatar, Brück, and Asch 2016), indicating that it may serve as a useful proxy for WUE_{wp} in early stages of development. Further, leaf biomass is highly associated with leaf area which can be rapidly screened via field-based imaging with unmanned aerial vehicles (see Roth et.al. 2018, Valle et.al. 2017, and Vadez et.al. 2015), providing opportunities to further develop high-throughput phenotyping of WUE_{wp} . However, these measures would need to account for variation in the relationship between leaf area and leaf biomass evident here.

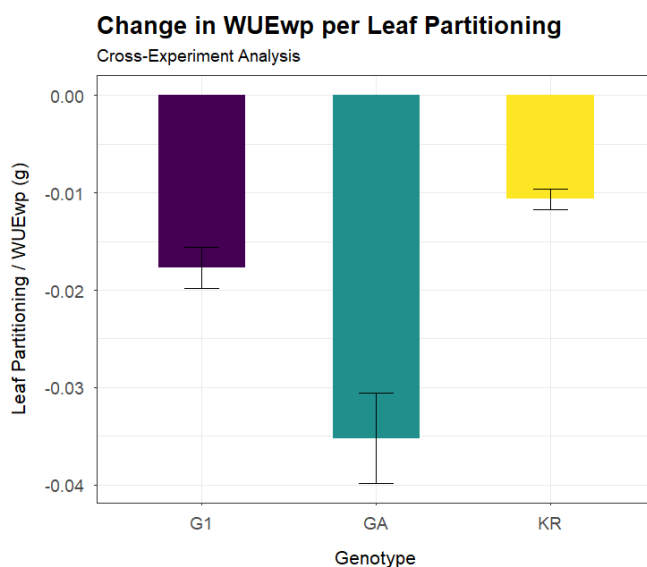


Figure 20: Bars represent the slope of the relationship between leaf partitioning and WUE_{wp} across all experiments in G1, GA, and KR +/- the standard error.

Genotypes G1 and KR consistently showed average to high mean WUE_{wp} , while this was significantly lower in GA. The relationship between WU and BM (i.e. WUE_{wp}) within

these genotypes was maintained in the cross-experiment analysis, where a two-fold difference was observed between the high-ranking genotypes KR and G1 and the low-ranking genotype GA. Such evidence indicates that any lack of variation in BM per WU was associated with either developmental or environmental differences as previously noted. Additionally, KR partitioned less biomass to the leaves in contrast to GA, which partitioned the most biomass to leaves. The impact of LP on WUE_{wp} varied between genotypes across all experiments (Figure 20). Increased LP in the genotype GA showed the most detrimental effect on WUE_{wp} , while in KR it showed the least. Thus, higher levels of leaf partitioning have a negative impact on WUE_{wp} across wheat genotypes, however the magnitude of this impact is genotype dependent. Thus, genotypes must differ in the relative contribution of leaf tissue to biomass gain and/or water use, through their leaf level regulation.

Genotypic variation WUE_i and Leaf Level Measures

Consistent with the literature (Wu and Boa 2011, Scotti-Campos et.al. 2015, Changhai et.al. 2010), minimal variation in WUE_i was observed across and within experiments as indicated by the relationship of A to g_s . Where WUE_i did vary in experiment 3, it contrasted with the measures of WUE_{wp} with KR having the lowest WUE_i and GA the highest (i.e. less and more change in A per g_s respectively). Initially, the response was attributed to variation in WUE_i across leaf age, as previously observed in wheat (Chen et.al. 2013, Atkinson et.al. 1989). However, no changes in WUE_i between leaf ages were apparent when examined within each genotype. Therefore, it is likely that measuring WUE_i across leaf age at the presented scale only partially predicts how leaves are responding across the whole plant. Further, when methods were repeated in humidity-controlled environments, no variation was detectable. The disparity between these environments is suspected to be the result of variation in canopy microclimate, which would lead to inconsistent WUE_i throughout the whole plant as shown in grapevines (Medrano et.al 2015, Medrano et.al 2012). Such leaf level variability is likely in wheat, as genotypes have shown differential sensitivity to changing VPD (Schoppach and Sadok 2012, Jaugueri et.al. 2018, Xue et.al. 2004) but has yet to be comprehensively examined.

Assimilation and stomatal conductance consistently varied between genotypes in experiments 2 and 3, with KR displaying the highest levels regardless of treatment. However, genotypes did not vary for the relationship of A or g_s to GWC, thus noted variability in these measures must have been associated with differences in GWC or their underlying biochemical limitation. Still it is possible that the narrower span of GWC in later experiments prevented detection of variation, thus expansion of the soil moisture gradient and gene pool would result in detection of differential stomatal sensitivity to soil drying as previously seen in wheat (Schoppach and Sadok 2012). Further, KR showed no decline in assimilation across the three measurement weeks whereas GA did, indicating differential ability to sustain A across leaf age. Decline in A across wheat leaf age has previously been noted in the third leaf (Atkinson et.al. 1989) and the flag leaf (Carmo-Silva et.al. 2016). In flag leaves the decline in A from pre to post anthesis varied between genotypes (Carmo-Silva et.al. 2016), although such a response has scarcely been

documented in early vegetative wheat leaves. Therefore, it is likely that differential ability to maintain A as leaves age across multiple plant development stages results in genotypic variation in contribution to biomass gain and yield.

Stomatal closure strongly limited C_i via restrictions in CO_2 through the stomatal pores, as apparent from their positive correlation across experiments. Both measures had a consistent negative effect on WUE_i , where increases in both C_i and g_s resulted in decline in WUE_i . Such response is a result of increased water loss and carbon gain as stomata open (Jauregui et.al. 2018, Wu and Boa 2011, Gilbert et. al. 2010, Lawlor 2002, Atkinson et.al. 1989). Additionally, the relationship of WUE_i and g_s varied across leaf age in experiment 3 but not 4, further suggesting that variation in daily environmental conditions and microclimate impacts measurements of WUE_i .

The canopy microclimate, but also partially leaf age, affect measurements of WUE_i and its associated parameters (A and g_s). By measuring across a longer period and multiple leaves to ensure physiologically older leaves, changes in WUE_i and g_s may have occurred. However, maintenance of A as leaves age seems to be associated with high WUE_{wp} , as observed in KR. It is possible that phytohormones may play a pivotal role in regulating decline in A across leaf age via changes in patterns of leaf senescence.

The role of Phytohormones in regulating WUE_i

Little variation exists for $[\text{ABA}]_i$ across wheat genotypes, where water deficit produced different levels of increase only in experiment 2 and leaf age had no impact. Further, the relationship of $[\text{ABA}]_i$ to g_s was consistent across genotypes, indicating that stomata are equally sensitive to ABA contrary to what has been previously reported (Saradadevi et.al. 2017, Saradadevi et.al. 2014). Variation in increase of $[\text{ABA}]_i$ in response to soil drying in experiment 2 may be due to variation in ABA production, translocation, or degradation. Differences in stomatal conductance (as noted above) may then be due to variation in GWC (or ABA at a given GWC) at time of measurement. However, further examination of variation in the mechanisms of ABA perception and production are required in order to determine the hormones role in regulation of WUE_i and WUE_{wp} under water deficit in wheat.

In vivo measurements of photosynthetic limitations

J_{max} and V_{cmax} have previously been shown to vary across wheat and soybean genotypes (Carmo-Silva et.al. 2016, Gilbert et.al. 2010), as was also evident here. The limitation of photosynthesis by rubisco is represented by V_{cmax} and limitation of photosynthesis by RUBP by J_{max} , where higher levels indicate lower limitation. Both measures were highest in KR, which also showed high WUE_{wp} . Further G1 displayed low V_{cmax} and J_{max} , corresponding to greater limitations and a comparatively lower WUE_{wp} in the same experiment. These measures were previously associated with higher achievable rates of photosynthesis (A_{max}) (Carmo-Silva et.al. 2016), consistent with results presented here where KR shows higher A , V_{cmax} , and J_{max} . However, as data is unavailable for GA, a low performing genotype, it is difficult to determine if they can be used to predict across a

range of WUE_{wp} and yield. Additionally, G1 displayed high levels of WUE_{wp} in the cross-experiment analysis, indicating value in measuring photosynthetic limitation across variable environments to increase accuracy in predicting WUE_{wp} . Further, no non-stomatal limitations were observed when these genotypes were exposed to soil water deficit, indicating that decline in A and WUE_i are a result of stomatal limitation consistent with what has been previously reported (Perdomo et.al. 2017).

The relationship between WUE_{wp} and WUE_i

It is still unclear how exactly to adjust measurement of WUE_i to more accurately predict WUE_{wp} , as these measures and their associated parameters showed little correlation within experiments but did in the cross-experiment analysis (Figure 21). Despite, other useful predictors have been established. Sustained assimilation as leaves age, as seen in KR, shows promise as a predictor of WUE_{wp} despite a lack of association with higher biomass gain. Decreased assimilation across leaf age with maintenance of stomatal conductance, as seen in GA, would result in water loss with no benefit to carbon fixation and thus biomass gain, negatively affecting WUE_i and WUE_{wp} . It would then be useful to expand the number of leaf positions and ages measured to assess if these results are consistent across the plant and canopy microclimate. Further, it is possible that such declines would limit yield, especially under water deficit where A is already limited by decline in g_s ; however, these relationships were not examined here.

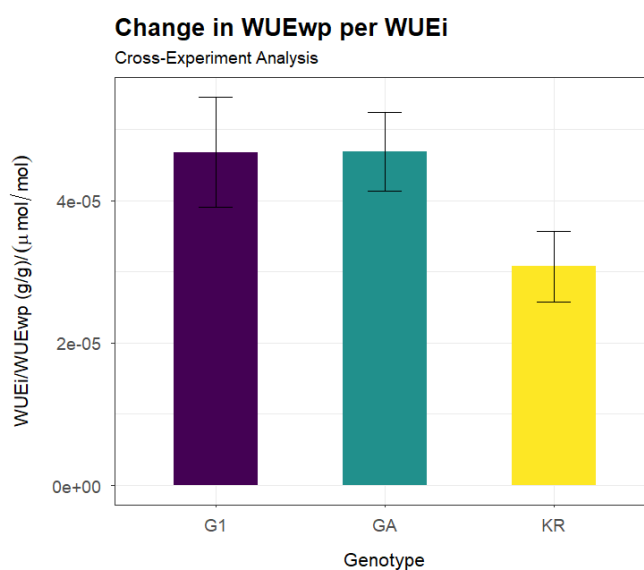


Figure 21: Bars represent the slope of the relationship between WUE_{wp} and WUE_i +/- the standard error.

The genotype KR, despite its high A and g_s , showed lower levels of WU across all experiments with no stable variation in BM. While such a result seems inconsistent, it is likely due to differences in the level of LP and LB. Less leaf matter would lead to less water lost via transpiration and less biomass gained via photosynthesis, despite a higher stomatal conductance (Figure 22). This seems probable as the genotype GA had low g_s and A , low WU and BM, and the highest levels of leaf partitioning and leaf biomass

(Figure 22). Here, lower g_s results in similar levels of WU between KR and GA due to greater transpiring leaf tissue.

Further, the correlation of LB and LP to LA provides an opportunity to develop models to predict WUE_{wp} through the association of these measures. However, LA was weakly correlated to WUE_{wp} which may be due to genotypic variation in leaf anatomy, where differences in leaf thickness, mesophyll distribution and conductance, and stomatal patterning may impact WUE_i and WUE_{wp} (Franks et. al. 2015, Tanaka et.al. 2013, Han et.al. 2016, Tomas et.al. 2014, Dow, Berry, and Bergmann 2017).

Additionally, it seems that differential regulation of RWC existed between genotypes. While soil drying decreased RWC in all genotypes, stomatal closure was correlated with RWC only in GA and KR. Further, GWC was correlated to g_s in all genotypes. However, increased $[ABA_i]$ was correlated to decreased GWC and g_s in G1 and KR, but not GA. Such evidence would suggest variation in the mechanisms regulating ABA production and perception, stomatal conductance, and water use across soil drying. Differential ability to maintain Ψ_1 (a proxy of RWC) has been previously noted in wheat (Saradadevi et/al 2014, Quarrie and Jones 1979), however higher Ψ_1 corresponded with lower levels of ABA and higher g_s (Quarrie and Jones 1979). It is possible that chemical signaling induced stomatal closure in some genotypes (KR and G1) while hydraulic signaling is utilized in others (GA) (Wilkinson and Davies 2010, Cornstock 2001, Shatil-Cohen et.al. 2013, Pantin et.al.2013, Sade et.al. 2014). However, no such mechanistic variation has yet been reported in wheat or other monocots.

Key Take-aways:

- Carbon assimilation (A) declines before stomatal conductance (g_s) as leaves age, resulting in inefficient water use
- Sustainability of A across leaf age varies between genotype, where genotypes that sustain A longer have higher WUE_{wp}
- Increased leaf partitioning and biomass result in greater water use, and are associated with lower WUE_{wp}
- Lower metabolic limitation to A is associated with high WUE_{wp}
- WUE_i and WUE_{wp} are significantly correlated, but only when examined in multiple environments

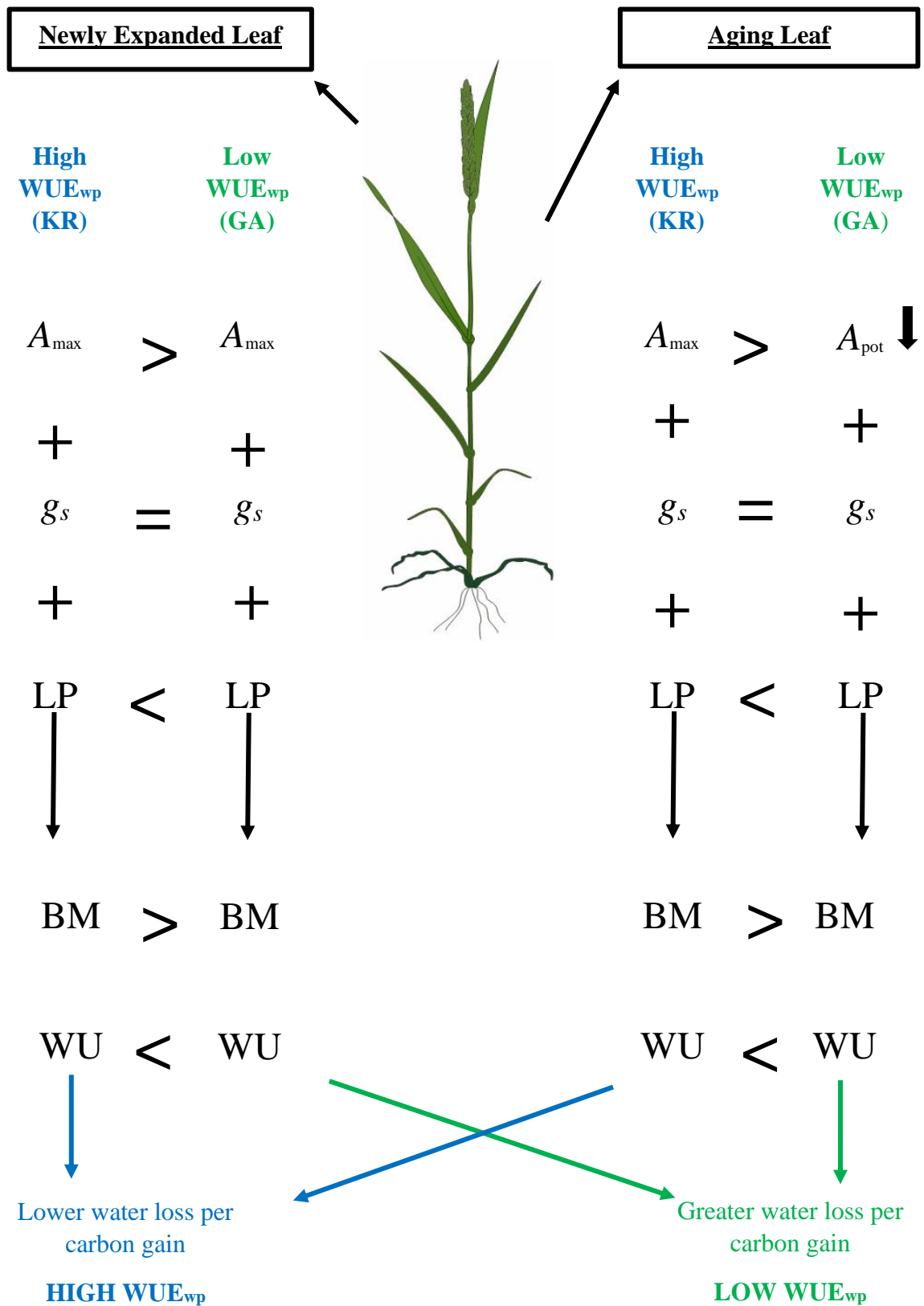


Figure 22: Diagram representing pathway of achieving whole plant phenotype of two contrasting genotypes. A_{max} = to the maximum assimilation rate, A_{pot} = potential assimilation rate under leaf aging, g_s = stomatal conductance, LP = leaf partitioning, BM = biomass gain, and WU = water use

Conclusion and Future Work:

Repeatable variation of WUE_{wp} exists among spring wheat genotypes, however this is not aligned with variation in WUE_i . The disconnect between these measures was likely a result of variation in leaf level measurements in response to canopy microclimate (Medrano et.al. 2015) as WUE_i and WUE_{wp} were correlated across experiments. Nevertheless, useful measures have been identified for predicting WUE_{wp} and tolerance to water deficit in wheat. Ability to sustain photosynthesis across leaf age, lower leaf biomass partitioning lower V_{cmax} and J_{max} , and lower stomatal limitation all lead to improved WUE_{wp} and should be assessed using presently available high-throughput techniques. The regulation of these traits can be further assessed through examining the impact of phytohormones (ABA and ACC) on stomatal conductance and assimilation across leaf age. Additionally, the limitation imposed by rubisco should be quantified to determine the biochemistry underlying maintenance of photosynthesis across leaf age. It is likely that the phytohormones are interacting with inherent photosynthetic biochemistry via regulation of senescence and thus enzyme activity as leaves age. Genotypic variation in these interactions would lead to differential leaf contribution to the whole plant phenotype.

Future work in this area should emphasise measuring assimilation and stomatal conductance across a variety of environmental conditions, such as gradients of soil moisture and vapour pressure deficit, as these stresses frequently co-occur. To determine how these responses vary across time and space, it would be useful to assess a greater number of leaf ages and positions. Additionally, *in vitro* measurement of photosynthetic enzymes could potentially validate the *in vivo* measurements presented here. Experiments described here-in could be replicated and taken to yield in order to correlate these measurements to crop productivity under optimal and deficit irrigation. Such experimentation would improve knowledge of early indicators of yield and enhance selection efficiency in plant breeding.

Acknowledgements:

It is important to identify those individuals who made all the presented work possible. Primarily, my supervisors Ian Dodd and Elizabete Carmo-Silva, whose tutelage and support permitted me and the project to excel. Additionally, I am grateful for all the guidance provided by members of both the plant-water stress and photosynthesis lab groups at Lancaster University. Specifically, Jaime Puértolas, Cristina Sales, Noorliana Mohd Zan, and Samuel Taylor who shared their expertise and taught me skills that will be useful throughout my scientific career. Additionally, I am indebted to Diego Zamudio Ayala and Dennise Patricia Deza Montoya who made the fourth experiment of the project possible. As visiting scientists, they took time out of their busy schedules to assist me with techniques they learned on site. Lastly, I would like to thank all the women in my life who remind me every day that not only are difficult things worth doing, but worth doing well.

Appendix A

Preliminary Experiments

Soil Drained Capacity

To determine how much water the plant growth substrate held, an initial experiment was conducted with soil-filled pots. Eight two-liter pots were weighed, filled with a mix of 3:1 (V:V) Petersfield compost and silver sand to 1 to 2 cm below the pot rim and saturated until water dripped from the base. Pots drained for a 16h period (17:00 to 9:00), after which pots were weighed at field capacity. The volumetric water content of the soil in each pot was also determined via three measurements with a soil moisture probe (theta probe, Delta-T Devices, Burwell, UK). Soil from each pot was then dried in an oven at 105°C for 3 days, and the soil was weighed once more. The amount (weight) of water in a pot at drained capacity was determined by subtracting the weight of oven dried soil plus pot weight from the weight of saturated pots. Knowing the volume of water within a pot at drained capacity improves the accuracy of estimating soil water content throughout the experiments.

<i>Pot #</i>	<i>Pot weight</i>	<i>Saturated weight + pot</i>	<i>Average Probe Read</i>	<i>Dry weight (no pot)</i>	<i>Water at 100% DC</i>
1	67.8	2114.2	31.8	1238.2	808.2
2	67.2	2247.9	34.7	1364.3	883.6
3	69.7	2293.2	61.5	1370.1	923.1
4	70	2237.3	37.8	1413.9	823.4
5	67.7	2171.4	43.2	1353.5	817.9
6	68.8	2267.2	38.0	1344.2	923.0
7	68.5	2008.4	61.1	1211.8	796.6
8	68.8	2066.9	44.4	1210.8	856.1
AVG	68.6	2175.8	44.1	1313.4	854.0

Table A.1: All weights were measured in grams, averages are standard mean values

Diurnal patterns in stomatal conductance

Stomatal conductance varies with numerous abiotic conditions including light intensity, temperature, and relative humidity. As these parameters fluctuate throughout the day, stomatal conductance changes as the day progresses. It is best to measure stomatal conductance when this is optimum to have the best reading. To measure this two of the replicates of each genotype within the WW treatment were measured for stomatal conductance at 9:00, 11:00, 13:00, 15:00 and 17:00 on a day where water was not administered. Results are displayed in Figure A.1 below and indicate that optimum measurement window occurs between 9:00 and 11:00 although reliable measurements may still be gathered before 13:00. Porometers were only used in the first experiment as more accurate equipment became available for use (see methods)). A measurement time from 9:00 to 13:00 was used throughout the first experiment.

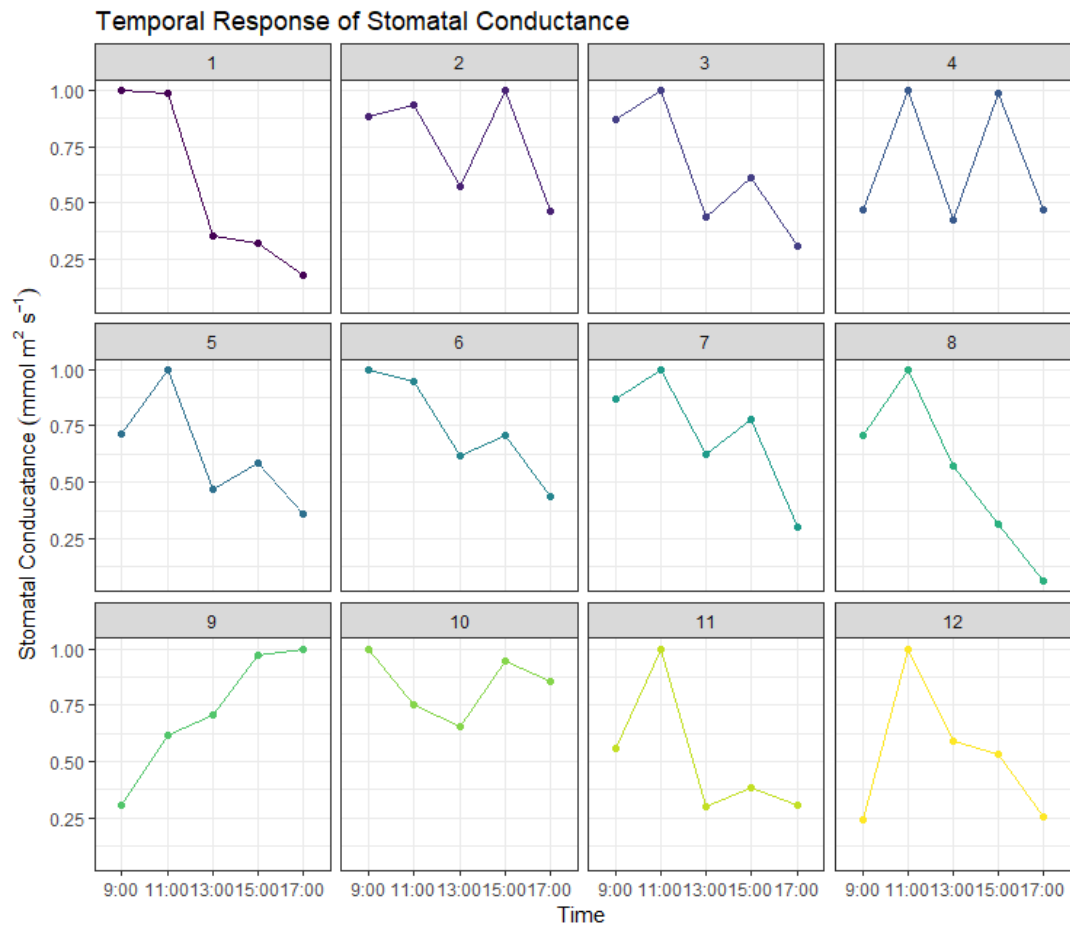


Figure A.1: Temporal response of stomata between 12 randomly selected plants. Stomatal conductance was normalized to the maximum value for each plant. Measurements were conducted with an AP4 porometer (Delta T Devices – Cambridge, UK).

Effects of re-watering on stomatal conductance

Stomatal conductance is thought to rapidly recover following re-watering. In order to determine the best time to measure conductance in relation to re-watering, stomatal conductance was measured in increments surrounding the watering time. Measurements were taken 1 hour before, 30 minutes before, 30 minutes after, 1 hour after, and 2 hours after re-watering for one replicate of each genotype. Knowing the stomatal response to re-watering aids in more accurate timing of measurement as well as proper analysis of data. No clear impact of re-watering was observed, so for the remainder of experiment plants were measured both before and after watering within a 30 minute to 1-hour period.

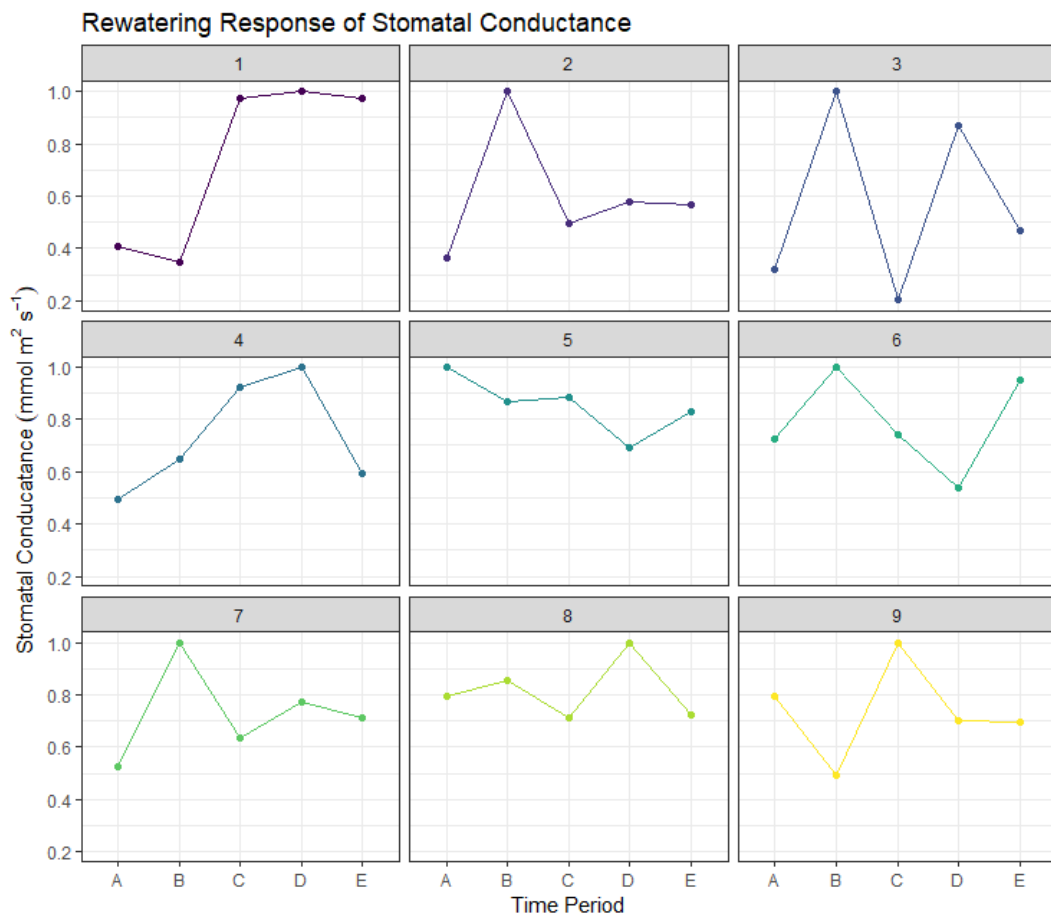


Figure A.2: Re-watering response of stomata between 9 randomly selected plants across five time points. A = 1-hour pre-watering, B = 30 minutes pre-watering, C = 30 minutes post-watering, D = 1 hour post-watering, E = 2-hours post-watering.

Stomatal conductance was normalized to the maximum value for each plant. Measurements were conducted with an AP4 porometer (Delta T Devices – Cambridge, UK).

Appendix B

All tables in this appendix indicate the p-values for the impact of genotype, treatment, and the interaction of the two on the specified measurement. Tables also include the r^2 value for these relationships, indicating the proportion of variability in the measurements accounted for by genotype and treatment. In experiment 1 and 2 the treatment was the different watering regiments (WW/WD). In experiments 3 and 4 the treatment was leaf age (weeks 1-3).

Table B.1

Experiment 1 p-values from ANOVA				
Measurement	<i>Genotype p-value</i>	<i>Treatment p-value</i>	<i>Genotype x Treatment p-value</i>	R^2
Biomass	1.150×10^{-1}	8.606×10^{-11}	3.023×10^{-1}	0.6777
Water Use	6.102×10^{-2}	4.873×10^{-13}	4.0901×10^{-1}	0.7358
WUE _{wp} (mean)	4.155×10^{-5}	6.810×10^{-1}	5.979×10^{-1}	0.5419
WUE _{wp} (trend)	6.078×10^{-6}	4.476×10^{-1}	8.243×10^{-1}	0.9906
Assimilation	4.237×10^{-1}	2.402×10^{-5}	5.697×10^{-1}	0.4527
Stomatal conductance (LICOR)	1.991×10^{-9}	5.109×10^{-1}	7.617×10^{-1}	0.6025
Stomatal Conductance (AP4)	2.444×10^{-5}	$< 2.2 \times 10^{-16}$	5.326×10^{-1}	0.4062
Intercellular CO ₂	0.0332	1.329×10^{-13}	2.423×10^{-1}	0.7549
WUE _i (mean)	1.984×10^{-1}	1.974×10^{-12}	4.101×10^{-1}	0.7131
WUE _i (trend)	5.69×10^{-2}	7.736×10^{-4}	2.60×10^{-3}	0.9405
Leaf Area	3.597×10^{-7}	$< 2.2 \times 10^{-16}$	1.42×10^{-3}	0.8865
Leaf Biomass	1.053×10^{-5}	6.235×10^{-14}	1.395×10^{-2}	0.8081
Leaf Partitioning	$< 2 \times 10^{-16}$	2.565×10^{-2}	3.7764×10^{-1}	0.8621
ABA	6.403×10^{-1}	1.422×10^{-1}	3.066×10^{-1}	0.2833

Table B.2

<i>Experiment 2 p-values from ANOVA</i>				
Measurement	<i>Genotype p-value</i>	<i>Treatment p-value</i>	<i>Genotype x Treatment p-value</i>	<i>R²</i>
Biomass	1.347×10^{-4}	$< 2.2 \times 10^{-16}$	2.357×10^{-5}	0.8615
Water Use	3.82×10^{-2}	$< 2.2 \times 10^{-16}$	5.201×10^{-2}	0.7222
WUE _{wp} (mean)	4.942×10^{-6}	6.877×10^{-3}	4.20×10^{-1}	0.4093
WUE _{wp} (trend)	1.478×10^{-6}	6.397×10^{-1}	9.236×10^{-1}	0.9534
Assimilation	1.477×10^{-9}	$< 2.2 \times 10^{-16}$	4.414×10^{-1}	0.4057
Stomatal conductance	6.377×10^{-10}	$< 2.2 \times 10^{-16}$	5.416×10^{-1}	0.3037
Assimilation Rebound	2.053×10^{-1}	4.908×10^{-1} [#]	3.554×10^{-1}	0.1868
Intercellular CO ₂	2.379×10^{-2}	3.612×10^{-1}	1.152×10^{-3}	0.0617
WUE _i (mean)	1.568×10^{-2}	1.717×10^{-12}	4.06×10^{-2}	0.1387
WUE _i (trend)	8.922×10^{-1}	3.744×10^{-2}	1.577×10^{-1}	NA ^{##}
Leaf Area	1.299×10^{-8}	$< 2.2 \times 10^{-16}$	6.998×10^{-6}	0.7963
Leaf Biomass	2.896×10^{-15}	$< 2.2 \times 10^{-16}$	2.621×10^{-8}	0.8774
Leaf Partitioning	$< 2.2 \times 10^{-16}$	5.282×10^{-7}	2.809×10^{-2}	0.9021
ABA	7.824×10^{-2}	6.146×10^{-4}	3.048×10^{-1}	0.2783

[#] Treatment refers to the re-watering cycle (1 through 3) for which rebound was calculated for (not WW/WD)

^{##} A mix model was used to fit the trend, R² is not provided in the summary. For this fit the p-value was $< 2.2 \times 10^{-16}$

Table B.3

Experiment 3 p-values from ANOVA				
Measurement	Genotype <i>p-value</i>	Treatment <i>p-value</i> [#]	Genotype x Treatment <i>p-value</i>	R ²
Biomass	2.5556×10^{-6}	1.087×10^{-6}	1.528×10^{-2}	0.7806
Water Use	3.091×10^{-4}	3.342×10^{-15}	1.021×10^{-5}	0.9159
WUE _{wp} (mean)	9.203×10^{-7}	6.6×10^{-1}	6.521×10^{-1}	0.6691
WUE _{wp} (trend)	7.014×10^{-1}	5.287×10^{-1}	2.974×10^{-1}	0.9222
Assimilation	6.432×10^{-8}	5.023×10^{-10}	2.23×10^{-1}	NA ^{##}
Stomatal conductance	4.728×10^{-14}	1.825×10^{-2}	4.147×10^{-1}	NA ^{##}
Intercellular CO ₂	4.636×10^{-10}	9.542×10^{-4}	1.984×10^{-1}	NA ^{##}
WUE _i (mean)	1.057×10^{-10}	2.332×10^{-2}	3.035×10^{-1}	NA ^{##}
WUE _i (trend)	1.339×10^{-2}	6.259×10^{-1}	7.481×10^{-1}	NA ^{##}
Spike Partitioning	6.165×10^{-9}	7.411×10^{-1}	3.752×10^{-1}	0.8375
Spike Number	7.622×10^{-1}	1.32×10^{-3}	4.698×10^{-1}	0.4297
Weight per Spike	2.262×10^{-3}	3.74×10^{-1}	5.199×10^{-1}	0.4709
ABA	5.382×10^{-1}	8.111×10^{-1}	6.334×10^{-1}	0.1121
V _{cmax}	9.124×10^{-4}	3.312×10^{-2}	2.518×10^{-1}	0.7229
J _{max}	3.471×10^{-2}	9.52×10^{-3}	3.426×10^{-1}	0.6103

[#] Treatment refers to either leaf age for leaf level measurements (WUE_i, C_i, A, and g_s) and refers to watering treatment for whole plant measures

^{##} A mix model was used to fit the trend, R² is not provided in the summary

Table B.4

Experiment 4 p-values from ANOVA				
Measurement	<i>Genotype</i> <i>p-value</i>	<i>Treatment</i> <i>p-value</i> [#]	<i>Genotype x</i> <i>Treatment</i> <i>p-value</i>	<i>R</i> ²
Biomass	7.099×10^{-5}	NA	NA	0.2581
Water Use	9.323×10^{-2}	NA	NA	0.07146
WUE _{wp} (mean)	9.213×10^{-6}	NA	NA	0.304
WUE _{wp} (trend)	5.616×10^{-1}	NA	NA	0.702
Assimilation	8.205×10^{-1}	7.109×10^{-3}	2.719×10^{-1}	NA ^{##}
Stomatal conductance	6.446×10^{-1}	4.438×10^{-1}	2.834×10^{-2}	NA ^{##}
Intercellular CO ₂	9.717×10^{-1}	3.133×10^{-5}	8.895×10^{-2}	NA ^{##}
WUE _i (mean)	9.892×10^{-1}	2.801×10^{-4}	4.142×10^{-2}	NA ^{##}
WUE _i (trend)	6.856×10^{-1}	2.839×10^{-1}	1.708×10^{-1}	NA ^{##}
Leaf Biomass	3.072×10^{-8}	NA	NA	0.4176
Leaf Partitioning	$< 2.2 \times 10^{-16}$	NA	NA	0.8785
ABA	9.248×10^{-1}	5.1×10^{-1}	3.34×10^{-1}	0.1605

[#] Treatment refers to leaf age, no other treatments were administered hence NA values for whole plant measurements

^{##} A mix model was used to fit the trend, R² is not provided in the summary

Appendix C

Table in this appendix indicate the Pearson's correlation coefficient and the associate P value between two specified traits.

Table C.1

<i>Experiment 1 Pearson's Correlation Values</i>			
<i>Measurement 1</i>	<i>Measurement 2</i>	<i>Correlation Coefficient</i>	<i>p-value</i>
<i>Biomass</i>	<i>Water Use</i>	0.945862	$< 2.2 \times 10^{-16}$
<i>Biomass</i>	<i>Assimilation</i>	0.3663206	3.15×10^{-3}
<i>Biomass</i>	<i>WUE_{wp}</i>	0.5196384	1.276×10^{-5}
<i>Water Use</i>	<i>WUE_{wp}</i>	0.2564859	4.245×10^{-2}
<i>Stomatal Conductance</i>	<i>Water Use</i>	0.4792007	7.088×10^{-5}
<i>Stomatal Conductance</i>	<i>Assimilation</i>	0.7076499	8.897×10^{-11}
<i>Stomatal Conductance</i>	<i>WUE_i</i>	-0.9078327	$< 2.2 \times 10^{-16}$
<i>Stomatal Conductance</i>	<i>Intercellular Carbon (C_i)</i>	0.8030031	2.443×10^{-15}
<i>Assimilation</i>	<i>Intercellular Carbon (C_i)</i>	0.7094946	7.562×10^{-11}
<i>Assimilation</i>	<i>WUE_i</i>	-0.6407963	1.543×10^{-8}
<i>Leaf Biomass</i>	<i>Water Use</i>	0.8990801	$< 2.2 \times 10^{-16}$
<i>Leaf Biomass</i>	<i>Leaf Area</i>	0.9415979	$< 2.2 \times 10^{-16}$
<i>Leaf Area</i>	<i>Water Use</i>	0.8925831	$< 2.2 \times 10^{-16}$
<i>Leaf Area</i>	<i>WUE_{wp}</i>	0.01967703	8.783×10^{-1}
<i>Leaf Partitioning</i>	<i>WUE_{wp}</i>	-0.7234803	2.114×10^{-11}
<i>Stomatal Conductance</i>	<i>Foliar ABA</i>	-0.463273	1.304×10^{-2}
<i>Foliar ABA</i>	<i>Soil Moisture</i>	-0.3801155	4.601×10^{-2}
<i>WUE_i</i>	<i>WUE_{wp}</i>	0.05388345	6.749×10^{-1}

Table C.2

Experiment 2 Pearson's Correlation Values			
Measurement 1	Measurement 2	Correlation Coefficient	p-value
Biomass	<i>Water Use</i>	0.896387	$< 2.2 \times 10^{-16}$
Biomass	WUE_{wp}	0.2992856	6.99×10^{-3}
Water Use	WUE_{wp}	0.01683716	8.822×10^{-1}
Stomatal Conductance	<i>Assimilation</i>	0.9274149	$< 2.2 \times 10^{-16}$
Stomatal Conductance	WUE_i	0.06538627	1.531×10^{-1}
Stomatal Conductance	<i>Intercellular Carbon (C_i)</i>	0.3397065	2.107×10^{-14}
Assimilation	<i>Intercellular Carbon (C_i)</i>	0.09142254	4.552×10^{-2}
Assimilation	WUE_i	0.2918395	7.374×20^{-11}
Leaf Biomass	<i>Water Use</i>	0.8664906	$< 2.2 \times 10^{-16}$
Leaf Biomass	<i>Leaf Area</i>	0.9291555	$< 2.2 \times 10^{-16}$
Leaf Area	<i>Water Use</i>	0.8247542	$< 2.2 \times 10^{-16}$
Leaf Area	WUE_{wp}	0.2777541	1.261×10^{-2}
Leaf Partitioning	WUE_{wp}	-0.05900258	6.031×10^{-1}
Stomatal Conductance	<i>Foliar ABA</i>	-0.4331108	6.697×10^{-5}
Foliar ABA	<i>Soil Moisture</i>	-0.6054222	4.309×10^{-9}
WUE_i	WUE_{wp}	0.3279332	3.174×10^{-3}

Table C.3

<i>Experiment 3 Pearson's Correlation Values</i>			
<i>Measurement 1</i>	<i>Measurement 2</i>	<i>Correlation Coefficient</i>	<i>p-value</i>
Biomass	<i>Water Use</i>	0.7713829	3.665×10^{-8}
Biomass	<i>Assimilation</i>	-0.06683395	6.985×10^{-1}
Biomass	<i>WUE_{wp}</i>	0.6736442	6.7×10^{-6}
Water Use	<i>WUE_{wp}</i>	0.1037625	5.47×10^{-1}
Stomatal Conductance	<i>Water Use</i>	-0.2232305	1.906×10^{-1}
Stomatal Conductance	<i>Assimilation</i>	0.7696503	$< 2.2 \times 10^{-16}$
Stomatal Conductance	<i>WUE_i</i>	-0.8307197	$< 2.2 \times 10^{-16}$
Stomatal Conductance	<i>Intercellular Carbon (C_i)</i>	0.7602222	$< 2.2 \times 10^{-16}$
Assimilation	<i>Intercellular Carbon (C_i)</i>	0.2287546	5.819×10^{-3}
Assimilation	<i>WUE_i</i>	-0.3435077	2.496×10^{-5}
Foliar ABA	<i>Stomatal Conductance</i>	0.1823417	1.397×10^{-1}
Foliar ABA	<i>Soil Moisture</i>	0.2555261	3.994×10^{-2}
Foliar ABA	<i>RWC</i>	-0.02045499	8.695×10^{-1}
Spike Partitioning	<i>WUE_{wp}</i>	0.2815636	1.548×10^{-1}
Spike Partitioning	<i>Water Use</i>	-0.1875438	3.489×10^{-1}
V_{cmax}	<i>J_{max}</i>	0.7990458	4.072×10^{-5}
WUE_i	<i>Intercellular Carbon (C_i)</i>	-0.9872387	$< 2.2 \times 10^{-16}$
WUE_i	<i>WUE_{wp}</i>	-0.2841208	9.308×10^{-2}

Table C.4

Experiment 4 Pearson's Correlation Values			
Measurement 1	Measurement 2	Correlation Coefficient	p-value
Biomass	<i>Water Use</i>	0.7325344	1.849×10^{-12}
Biomass	<i>Assimilation</i>	0.01017515	9.349×10^{-1}
Biomass	<i>WUE_{wp}</i>	0.9188591	$< 2.2 \times 10^{-16}$
Water Use	<i>WUE_{wp}</i>	0.4208631	3.907×10^{-4}
Stomatal Conductance	<i>Water Use</i>	0.1606286	1.941×10^{-1}
Stomatal Conductance	<i>Assimilation</i>	0.639431	2.552×10^{-7}
Stomatal Conductance	<i>WUE_i</i>	-0.5896013	3.395×10^{-6}
Stomatal Conductance	<i>Intercellular Carbon (C_i)</i>	0.4799086	2.765×10^{-4}
Assimilation	<i>Intercellular Carbon (C_i)</i>	-0.3262776	1.1711×10^{-2}
Assimilation	<i>WUE_i</i>	0.2002409	1.505×10^{-1}
Leaf Biomass	<i>Water Use</i>	0.7348376	1.453×10^{-12}
Leaf Biomass	<i>Biomass</i>	0.8117667	$< 2.2 \times 10^{-16}$
Leaf Biomass	<i>WUE_{wp}</i>	0.6689322	6.128×10^{-10}
Leaf Biomass	<i>Leaf Partitioning</i>	0.5463834	1.727×10^{-6}
Leaf Partitioning	<i>Water Use</i>	0.2290406	6.227×10^{-2}
Leaf Partitioning	<i>Biomass</i>	-0.02495164	8.411×10^{-1}
Leaf Partitioning	<i>WUE_{wp}</i>	-0.1522943	2.186×10^{-1}
Foliar ABA	<i>RWC</i>	0.1982225	1.194×10^{-1}
Foliar ABA	<i>Stomatal Conductance</i>	-0.1897969	1.363×10^{-1}
Foliar ABA	<i>Soil Moisture</i>	-0.2354918	6.54×10^{-2}
WUE_i	<i>Intercellular Carbon (C_i)</i>	-0.9834274	$< 2.2 \times 10^{-16}$
WUE_i	<i>WUE_{wp}</i>	0.2148596	8.08×10^{-2}

Table C.5

Cross Experiment Pearson's Correlation Values			
Measurement 1	Measurement 2	Correlation Coefficient	p-value
Gatsby			
Biomass	Water Use	0.8122625	3.006×10^{-14}
Biomass	Assimilation		
Stomatal Conductance	Water Use	0.3825244	3.62×10^{-3}
Stomatal Conductance	Assimilation	0.8590141	$< 2.2 \times 10^{-16}$
Leaf Biomass	Water Use	0.9315377	$< 2.2 \times 10^{-16}$
Leaf Biomass	Leaf Partitioning	-0.0138733	9.263×10^{-1}
Leaf Biomass	Biomass	0.9224475	$< 2.2 \times 10^{-16}$
Leaf Partitioning	Water Use	0.5773822	2.148×10^{-5}
Leaf Partitioning	WUE _{wp}	-0.7497699	1.321×10^{-9}
Biomass	WUE _{wp}	-0.1783401	1.885×10^{-1}
Water Use	WUE _{wp}	-0.596475	1.227×10^{-6}
WUE_i	RWC	-0.7116676	6.889×10^{-9}
WUE_i	Foliar ABA	0.1043374	4.617×10^{-1}
Stomatal Conductance	Foliar ABA	-0.2386767	8.838×10^{-1}
Foliar ABA	Soil moisture (GWC)	-0.1982931	1.588×10^{-1}
Stomatal conductance	Relative water content	0.4675283	6.187×10^{-4}
Stomatal Conductance	Soil moisture (GWC)	0.5816744	2.582×10^{-6}
WUE_i	Soil moisture (GWC)	-0.7273149	2.174×10^{-10}
Relative water content	Foliar ABA	-0.01077466	9.427×10^{-1}
Relative water content	Soil moisture (GWC)	0.8748481	$< 2.2 \times 10^{-16}$
Stomatal conductance	Intercellular Carbon (C _i)	0.7496084	2.987×10^{-11}
Assimilation	Intercellular Carbon (C _i)	0.5241407	3.383×10^{-5}
WUE_i	WUE _{wp}	0.7532482	2.118×10^{-11}
G1			
Biomass	Water Use	0.8962445	$< 2.2 \times 10^{-16}$
Biomass	Assimilation	0.08679617	5.247×10^{-1}
Stomatal Conductance	Water Use	0.1768969	1.922×10^{-1}

Stomatal Conductance	Assimilation	0.8123112	2.987×10^{-14}
Leaf Biomass	Water Use	0.9565978	$< 2.2 \times 10^{-16}$
Leaf Biomass	Leaf Partitioning	0.4494499	5.11×10^{-4}
Leaf Biomass	Biomass	0.9594331	$< 2.2 \times 10^{-16}$
Leaf Partitioning	Water Use	0.5737068	3.797×10^{-6}
Leaf Partitioning	WUE_{wp}	-0.7528051	2.209×10^{-11}
Biomass	WUE_{wp}	-0.0734098	5.908×10^{-1}
Water Use	WUE_{wp}	-0.408901	1.754×10^{-3}
WUE_i	RWC	-0.6160628	3.143×10^{-6}
WUE_i	Foliar ABA	0.5923743	4.666×10^{-6}
Stomatal Conductance	Foliar ABA	-0.4623456	6.361×10^{-4}
Foliar ABA	Soil moisture (GWC)	-0.5363422	4.976×10^{-5}
Stomatal conductance	Relative water content	0.2616041	7.248×10^{-1}
Stomatal Conductance	Soil moisture (GWC)	0.4850564	1.514×10^{-4}
WUE_i	Soil moisture (GWC)	-0.7613973	9.595×10^{-12}
Relative water content	Foliar ABA	-0.6170073	8.176×10^{-6}
Relative water content	Soil moisture (GWC)	0.8436439	$5.172e-14$
Stomatal conductance	Intercellular Carbon (C_i)	0.7191799	4.277×10^{-10}
Assimilation	Intercellular Carbon (C_i)	0.4821958	1.678×10^{-4}
WUE_i	WUE_{wp}	0.6354987	1.426×10^{-7}
Krichauff			
Biomass	Water Use		
Biomass	Water Use	0.8417437	8.337×10^{-16}
Biomass	Assimilation	0.3319593	1.329×10^{-2}
Stomatal Conductance	Water Use	0.4736836	2.594×10^{-4}
Stomatal Conductance	Assimilation	0.7841466	1.434×10^{-12}
Leaf Biomass	Water Use	0.9439422	$< 2.2 \times 10^{-16}$
Leaf Biomass	Leaf Partitioning	0.4006746	2.435×10^{-3}
Leaf Biomass	Biomass	0.9334155	$< 2.2 \times 10^{-16}$
Leaf Partitioning	Water Use	0.5643732	7.198×10^{-6}
Leaf Partitioning	WUE_{wp}	-0.8107294	6.319×10^{-14}
Biomass	WUE_{wp}	0.1665148	$2,243 \times 10^{-1}$
Water Use	WUE_{wp}	-0.3317906	1.334×10^{-2}
WUE_i	RWC	-0.3781726	9.561×10^{-3}
WUE_i	Foliar ABA	0.4809316	5.4×10^{-4}

<i>Stomatal Conductance</i>	<i>Foliar ABA</i>	-0.3852178	6.857×10^{-3}
<i>Foliar ABA</i>	<i>Soil moisture (GWC)</i>	-0.405855	5.135×10^{-3}
<i>Stomatal conductance</i>	<i>Relative water content</i>	0.3787653	9.439×10^{-3}
<i>Stomatal Conductance</i>	<i>Soil moisture (GWC)</i>	0.6320851	3.848×10^{-7}
<i>WUE_i</i>	<i>Soil moisture (GWC)</i>	-0.766739	2.181×10^{-11}
<i>Relative water content</i>	<i>Foliar ABA</i>	-0.02236885	8.868×10^{-1}
<i>Relative water content</i>	<i>Soil moisture (GWC)</i>	0.3800417	1.002×10^{-2}
<i>Stomatal conductance</i>	<i>Intercellular Carbon (C_i)</i>	0.781369	1.938×10^{-12}
<i>Assimilation</i>	<i>Intercellular Carbon (C_i)</i>	0.4762965	2.371×10^{-4}
<i>WUE_i</i>	<i>WUE_{wp}</i>	0.6476492	9.075×10^{-8}

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