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A genetic link between whole-plant water use efficiency and leaf carbon isotope composition in the C_4 grass *Setaria*

Short title: Water use efficiency and C_4 leaf carbon isotopes

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Abstract

Increasing whole plant water use efficiency (yield per transpiration; WUE_{plant}) through plant breeding can benefit the sustainability of agriculture and improve crop yield under drought. To select for WUE_{plant}, an efficient phenotyping method that reports on the genetic contribution of component traits such as transpiration efficiency (TE_i; rate of CO_2 assimilation per stomatal conductance) must be developed. Leaf carbon stable isotope composition ($\delta^{13}C_{leaf}$) has been proposed as a high-throughput proxy for TE_i, and a negative correlation between $\delta^{13}C_{leaf}$ and both WUE_{plant} and TE_i has previously been demonstrated in several C_4 grass species. Therefore, the aim of the research presented here was to determine if the same loci control $\delta^{13}C_{leaf}$, WUE_{plant}, and TE_i under well-watered and water-limited conditions in a recombinant inbred line (RIL) population of closely related C_4 grasses *Setaria viridis* and *S. italica*. Three quantitative trait loci (QTL) for $\delta^{13}C_{leaf}$ were co-localized with transpiration, biomass, and a linear model of WUE. When WUE_{plant} was calculated for allele classes based on the three QTL for $\delta^{13}C_{leaf}$, $\delta^{13}C_{leaf}$ was negatively correlated with WUE_{plant} as theory predicts when WUE_{plant} is in part driven by differences in TE_i. In any population, multiple traits can influence WUE_{plant}; however, the analysis of $\delta^{13}C_{leaf}$ in this RIL population demonstrates that there is genetic control of TE_i that significantly contributes to WUE_{plant}. Furthermore, this research suggests that $\delta^{13}C_{leaf}$ can be used in marker-assisted breeding to select for TE_i and as a tool to better understand the physiology and genetic architecture of TE_i and WUE_{plant} in C_4 species.

Significance Statement

Overextended water resources and drought are major agricultural problems worldwide. Therefore, selection for increased plant water use efficiency (WUE_{plant}) in food and biofuel crop species is an important trait in plant breeding programs. Leaf carbon isotopic composition ($\delta^{13}C_{leaf}$) has potential as a rapid and effective high throughput phenotyping method for intrinsic transpiration efficiency (TE_i), an important leaf-level component trait of WUE_{plant}. Our research shows that $\delta^{13}C_{leaf}$ and WUE_{plant} share a common genetic architecture through their shared relationship with TE_i. This suggests that $\delta^{13}C_{leaf}$ can be used as a screen for TE_i in marker-assisted plant breeding programs to improve crop drought resistance and decrease agricultural water consumption.

Introduction

Water availability constrains agricultural production and threatens food security in many drought-prone regions (1). Therefore, improving the harvestable yield relative to water supplied to crop systems (agronomic water use efficiency; WUE_{ag}) has long received attention from researchers and government agencies (2-5). It has been proposed by Passioura (2) that yield could be improved relative to available water by increasing (1) the ratio of transpiration (T) to evapotranspiration (ET), (2) whole plant water use efficiency (ratio of biomass production to total transpiration; WUE_{plant}), and (3) harvest index (HI). To date, increases in WUE_{ag} have been primarily made by improved management practices that increase T/ET through improved water application (6, 7), increased canopy cover (8) and mulching (6). Additionally, selecting for greater HI has also increased WUE_{ag}, for example, with semi-dwarf wheat varieties (9). Unfortunately, in many agricultural settings, these traits appear to have approached their theoretical maximum.

To date, there has been limited change to WUE_{ag} through selection for increased WUE_{plant}. This is primarily because WUE_{plant} is a complex trait and is influence by 1) CO_2 assimilation (A_{net}) relative to water loss via stomata conductance (g_s), (i.e. the intrinsic transpiration efficiency, A_{net}/g_s; TE_i), 2) the portion of carbon loss from whole plant respiration (ϕ_c), 3) "nonproductive" water loss from cuticular and nighttime transpiration (ϕ_w), and 4) the evaporative demand between the atmosphere and the plant (See theory; 10, 11, 12). Theoretically, the first three of these factors can be selected for through plant breeding, but these traits, especially ϕ_c and ϕ_w, are determined by a complex set of composite traits that are difficult to measure and select for in breeding programs (13-15). Alternatively, in theory, TE_i is an ideal trait to select for because it is independent of environmental conditions driving changes in evaporative demands (16, 17), and it is an important component of WUE_{plant} as it relates to both CO_2 and H_2O leaf exchange, influencing both photosynthetic capacity and T (15, 18). Unfortunately, the primary means of estimating TE_i with gas exchange measurements of A_{net}/g_s do not integrate over time and generally do not represent TE_i over the lifetime of the plant or even the leaf (18). Furthermore, these measurements are prohibitively time-consuming and laborious, making this method impractical for selecting for WUE_{plant} in a plant-breeding program.

Alternatively, leaf carbon isotope composition ($\delta^{13}C_{leaf}$) has long been promoted as a proxy for an integrated measurement of TE_i in C_3 and potentially in C_4 species (10-12, 19, 20). In C_3 plants, the relationship between $\delta^{13}C_{leaf}$ and TE_i has been tested and even integrated into breeding programs (12, 15, 18, 21-26). However, in C_4 species, it remains uncertain if $\delta^{13}C_{leaf}$ is an effective proxy for TE_i and if there is a genetic link between these two traits. Nonetheless, empirical evidence supporting the theoretical relationship between $\delta^{13}C_{leaf}$ and TE_i has been documented in multiple C_4 species such as *Setaria viridis*, *S. italica*, *Zea mays*, and *Sorghum bicolor* (27-29). These studies also demonstrated consistent differences in $\delta^{13}C_{leaf}$ between well-watered and water-limited plants that negatively correlated with TE_i. Additionally, in *S. viridis* and *S. italica*, TE_i correlated with WUE_{plant} (29). However, other studies on both C_3 and C_4 species have found that TE_i and WUE_{plant} were not correlated (30-40). Therefore, the contribution of TE_i to WUE_{plant} requires further investigations to delineate those factors that collectively contribute to WUE_{plant}.

The genetic loci controlling WUE_{plant} and its relationship to TE_i and $\delta^{13}C_{leaf}$ can potentially be identified using large mapping populations grown on automated phenotyping systems that measure plant water use and biomass accumulation on hundreds of individual plants (41, 42). In fact, quantitative trait loci (QTL) have been found for $\delta^{13}C_{leaf}$ in several C_3 species such as rice (43-45), barley (46), *Brachypodium distachyon* (47), wheat (48), tomato (49), Arabidopsis (50, 51), sunflower (52), soybean (53), cotton (54), *Quercus ruber* (55), and *Stylosanthes scabra* (56). Additionally, a few studies on C_3 plants have found co-localized QTL for $\delta^{13}C_{leaf}$ and WUE_{plant} (52, 57), and, in one case, $\delta^{13}C_{leaf}$ and TE_i were associated with a causal gene (ERECTA; 58). However, to date only one publication found that $\delta^{13}C$ was under genetic control in a C_4 species (maize; 59), but this was not tested in terms of WUE. Therefore, to effectively use marker-assisted breeding to select for WUE_{plant} and TE_i in C_4 plants requires a more thorough understanding of the physiological relationship and genetic architecture of $\delta^{13}C_{leaf}$, TE_i, and WUE_{plant}.

Here a recombinant inbred line (RIL) population of 189 lines created from accession A10 of *S. viridis* (L.) P. Beauv. and accession B100 of *S. italica* (L.) P. Beauv was used to screen for WUE_{plant}, TE_i, and $\delta^{13}C_{leaf}$ (42, 60, 61). Both *S. viridis* and *S. italica* are model C_4 grasses in the same panicoid clade as important C_4 crops such as maize, sugarcane, sorghum and emerging bioenergy crops Miscanthus and switchgrass. The

objectives of this study were to compare $\delta^{13}C_{leaf}$ between plants grown under well-watered and water-limited conditions and to determine the genetic and physiological relationship between WUE_{plant}, TE_i, and $\delta^{13}C_{leaf}$.

Results

Fresh biomass, transpiration, WUE_{ratio}*,* WUE_{fit}*, and* WUE_{res} *traits*

Although whole plant growth and water use were analyzed throughout the experiment (42) we selected day 25 for our analysis when most genotypes were in the vegetative stage. On this day, the fresh biomass estimated from side view images and validated with final harvest biomass (42) varied across genotypes from 3.70 to 29.00 g and 1.40 to 10.30 g in the well-watered and water-limited treatments, respectively. Cumulative transpiration ranged from 367 to 1433 ml in the well-watered and 96 to 433 ml in water-limited plants. There was a significant difference in fresh biomass and transpiration rates between genotypes in both irrigation treatments (Fig. 1), and across genotypes the water-limited treatment significantly reduced fresh biomass and transpiration by 64 and 65 %, respectively (Table 1). The ratio of biomass relative to the amount of total plant transpiration (WUE_{ratio}) was 20 % higher in the water-limited treatment and ranged across genotypes from 1.2 to 36.7 (g/L) in the well-watered treatment and 3.9 to 41.7 (g/L) in the water-limited treatment, respectively (Table 1).

Across genotypes, output of the linear model of fresh biomass versus transpiration (WUE_{fit}) ranged from 1.84 to 36.24 g in the well-watered and 0.49 to 9.94 g in the water-limited treatments (Table 1). In the well-watered treatment, the residual of the WUE model (WUE_{res}) varied from -9.61 to 10.06 g and in the water-limited treatment from -6.22 to 2.25 g, with a significant genotype effect within each treatment. Because WUE_{res} was calculated for each treatment separately and centered around 0, no difference was expected between treatments (Table 1).

Broad-sense heritability, proportional variance and leaf carbon isotopic composition ($\delta^{13}C_{leaf}$*)*

In all traits, except WUE_{res}, 32 to 86 % of the variance in the experiment was explained by the treatment effect (Table 2). Additionally, in all traits, except WUE_{ratio} and WUE_{res}, the variance ascribed to the genotype effect was relatively small but substantial given the large influence that the water limitation treatment had on these traits.

Broad-sense heritability (H^2) was relatively robust for all traits, including $\delta^{13}C_{leaf}$, in at least one treatment or when treatments were combined (Table 2). For example,

$\delta^{13}C_{leaf}$ was significantly heritable in the well-watered treatment but not in the water-limited treatment. This is true of other traits, except WUE_{res}, were the well-watered treatment had higher H^2 than the water-limited treatment or combined treatments. The decrease of H^2 in the water-limited compared to the well-watered treatment was pronounced in all traits except fresh biomass and WUE_{res}. The $\delta^{13}C_{leaf}$ values ranged from -14.7 to -12.4 ‰ in the well-watered and -15.6 to -13.2 ‰ in the water-limited treatment, with significant differences across genotypes (Fig. 1; Table 1). The water-limited treatment significantly reduced $\delta^{13}C_{leaf}$ on average across genotypes by 0.82 ± 0.04 ‰ (Table 1).

Correlation of traits with $\delta^{13}C_{leaf}$

Over the time course of the experiment, the correlations of $\delta^{13}C_{leaf}$ with transpiration, biomass, and WUE_{fit} were constant except for a few days at the beginning and end of the experiment, so at the midpoint of the experiment (day 25), correlations with $\delta^{13}C_{leaf}$ were similar in magnitude to most days of the experiment. These correlations were stronger across treatments than within treatment, yet they were significant under the well-watered conditions (Fig. S1). On day 25, the correlation coefficients of $\delta^{13}C_{leaf}$ with fresh biomass, transpiration, and WUE_{fit} were between 0.51 and 0.61 across treatments and 0.30 to 0.33 for well-watered treatment (Fig. 1). For WUE_{ratio} and WUE_{res}, the correlation coefficients were low in both treatments and when the treatments were combined (Fig. S1).

QTL analysis and contributions of allele composition on traits

Three QTL (chr. 7@51 centimorgans (cM), chr. 7@99 cM and chr. 9@34 cM) associated with $\delta^{13}C_{leaf}$ were found in the well-watered treatment, but none were detected in the water-limited treatment (Table 3). All three of these QTL co-localized with WUE_{fit} and transpiration in both treatments on day 25 (as well as days 17 through 33 as described in Feldman, *et al.* (42). Two QTL (7@99 and 9@34) were also associated with fresh biomass in both treatments (Table 3). Furthermore, one QTL (9@34) of WUE_{ratio} was co-localized with $\delta^{13}C_{leaf}$. WUE_{res} were associated with two QTL (2@96 and 5@109) that co-localized with fresh biomass, transpiration, WUE_{ratio}, and WUE_{fit} in both treatments, but not with $\delta^{13}C_{leaf}$. Having an allele from parental accession A10 (*S. viridis*) at two of the three loci (7@51 and 7@99) increased $\delta^{13}C_{leaf}$ in the well-watered treatment, while an allele from parental line B100 (*S. italica*) increased $\delta^{13}C_{leaf}$ at 9@34 in both treatments (Fig. S2).

Combining alleles from the three QTL associated with $\delta^{13}C$ produced eight possible allele classes, and all but one (ABA) was present in this population. For six of the seven allele classes, the relationship between fresh biomass and transpiration was significant (Table 4; Fig. 2A). Furthermore, the regression of $\delta^{13}C_{leaf}$ against the slopes of fresh biomass versus transpiration showed a strong negative relationship for both the well-watered ($\delta^{13}C_{leaf}$ = -0.027 slope – 12.48; R^2 = 0.69; $P < 0.05$) and in the water-limited treatments ($\delta^{13}C_{leaf}$ = -0.012 slope – 13.82; R^2 = 0.80; $P < 0.05$; Fig. 2B). Although this relationship was dampened in the water-limited treatment, it followed a similar trend (Fig. 2B). Additionally, the order that the allele classes are positioned along the $\delta^{13}C_{leaf}$ versus slope regression is the same in both treatments. In the well-watered treatment, the QTL 7@99 (represented by the second letter in three-letter allele class names) appears to have the greatest influence on this relationship, where the A10 allele was associated with a reduced slope and enriched $\delta^{13}C_{leaf}$ (Fig. 2B). Alternatively, in the water-limited treatment, the effect of QTL 7@99 on this relationship was reduced relative to the well-watered treatment. Additionally, in both treatements the mean fresh biomass and transpiration for each of these allele classes had a strong significant positive relationship with $\delta^{13}C_{leaf}$ (Fig. 3A-B).

Discussion

Leaf carbon isotope composition ($\delta^{13}C_{leaf}$) has been theoretically related to TE_i (A_{net}/g_s) for both C_3 and C_4 species (12, 15, 18, 19, 27). However, $\delta^{13}C_{leaf}$ is less well-understood in C_4 species because the CO_2-concentrating mechanism dampens variability in leaf CO_2 discrimination and ultimately $\delta^{13}C_{leaf}$ (62). However, in this study we demonstrate significant genetic and environmental influence on $\delta^{13}C_{leaf}$ in a C_4 species that is driven by differences in water use efficiency. Although the variation in $\delta^{13}C_{leaf}$ in C_4 species is typically less than in C_3 species, in this study, a relatively large range in $\delta^{13}C_{leaf}$ (2.4 ‰) was observed across genotypes, and there was a significant mean 0.82 ± 0.04 ‰ difference between treatments, showing considerable genotypic by treatment response. These results are similar to previous studies of well-watered and water-limited C_4 plants (29, 63, 64), suggesting $\delta^{13}C_{leaf}$ in C_4 plants is both genetically determined and responsive to environmental conditions such as water limitation.

In the well-watered treatment, the positive correlations of fresh biomass, transpiration, and WUE_{fit} with $\delta^{13}C_{leaf}$ and similarities in the genetic architecture of these traits,i suggest that TE_i (as represented by $\delta^{13}C_{leaf}$) is important in determining the

amount of biomass produced for a given volume of water transpired. However, in the water-limited plants the low variation of $\delta^{13}C_{leaf}$, the lack of $\delta^{13}C_{leaf}$ correlations with other traits (e.g. *WUE_{ratio} and WUE_{res}*), and no QTLs for $\delta^{13}C_{leaf}$ was likely due to restricted stomatal conductance (g_s) across most genotypes, minimizing individual differences in TE_i, as found for C_3 species (52, 65). As stated previously, WUE_{res} represents how individuals deviate from WUE_{fit} either by A_{net}/g_s or other whole plant process. However, the lack of overlap in genetic architecture and the lack of correlation between WUE_{res} and $\delta^{13}C_{leaf}$ suggest that TE_i is not the primary driver of WUE_{res}. The major QTL associated with WUE_{res} (2@96, 5@109) may potentially allocate carbon to non-transpiring biomass such as structural and stem tissue (66). Additionally, the similar genetic architecture for WUE_{res} and WUE_{ratio}, indicating that in this population ϕ_w, ϕ_c, and r influence variation in WUE_{res} of individual plants relative to WUE_{fit} (42).

Three QTL of WUE_{fit} co-localized with $\delta^{13}C_{leaf}$ (7@51, 7@99, 9@34), suggesting that these loci are related to genotypic variation in TE_i. Based on the theoretical and empirical relationships, TE_i and $\delta^{13}C_{leaf}$ should be negatively correlated (20, 27, 62). Therefore, if TE_i is a major contributor to WUE_{plant} within an allele class, then WUE_{plant} should negatively correlate with $\delta^{13}C_{leaf}$. The WUE_{plant}, defined as the slope of the linear regression of fresh biomass versus transpiration (1, 67), was negatively correlated with $\delta^{13}C_{leaf}$ for each allele class. This suggests that $\delta^{13}C_{leaf}$ is genetically and physiologically related to WUE_{plant}, likely through TE_i. This relationship between the allele class-specific WUE_{plant} and $\delta^{13}C_{leaf}$ existed in the water-limited treatment as well, although no QTL for $\delta^{13}C_{leaf}$ were found. Given that the water limitation did not remove the underlying relationship between TE_i and WUE_{plant}, the inability to detect QTL is likely due to the reduced variation in $\delta^{13}C_{leaf}$, which reduced the magnitude of the genotypic response and decreased the signal to noise ratio. Alleles increasing WUE were contributed by both the weedy *S. viridis* (A10 parental accession) and the domesticated *S. italica* (B100 parental accession). In addition, allele classes followed the same order along the $\delta^{13}C_{leaf}$ versus slope regression in both well-watered and water-limited treatment blocks. For example, under both well-watered and water-limited conditions the allele class AAB had the lowest WUE_{plant} and most enriched $\delta^{13}C_{leaf}$; whereas allele classes BBB and BBA had the highest WUE_{plant} and most depleted $\delta^{13}C_{leaf}$ under both treatments. This trend indicates a strong allelic effect on relationship between $\delta^{13}C_{leaf}$, TE_i, and WUE_{plant} that will allow the selection for $\delta^{13}C_{leaf}$ to improve TE_i, WUE_{plant}, and drought tolerance.

Across allele classes, $\delta^{13}C_{leaf}$ also formed significant correlations with biomass and transpiration. A positive correlation between $\delta^{13}C_{leaf}$ and fresh biomass is expected if large plants have low TE_i and small plants have high TE_i. This scenario is possible, if the photosynthetic capacity (i.e. chlorophyll and Rubisco) are diluted across large leaves. This will decrease the photosynthetic rates per unit leaf area but on a per plant basis would remain large relative to small plants with fewer small leaves. In this case, the strong, positive relationship between $\delta^{13}C_{leaf}$ and transpiration would be driven by increased leaf area in the large plants. However, C_4 photosynthesis is mostly CO_2 saturated at ambient CO_2 concentrations, so variation in TE_i is likely due to variation in g_s across genotypes such that T decreases without negative impacts on A_{net}. This is further supported from studies of C_3 species that showed that g_s and not A_{net} varied with TE_i and $\delta^{13}C_{leaf}$ (67-70).

For $\delta^{13}C_{leaf}$ to be an effective screen for TE_i and WUE_{plant} of a C_4 species, it must show genotypic variation, be responsive to environmental conditions that influence TE_i, and be physiologically related to WUE_{plant}. In this C_4 grass RIL population, $\delta^{13}C_{leaf}$ showed significant and consistent response to water limitation, significant genotypic variation, and high heritability. Additionally, $\delta^{13}C_{leaf}$ correlated with transpiration, biomass, and the linear relationship between biomass and transpiration (WUE_{fit}), suggesting a physiological relationship among these traits. This is further supported by the fact that $\delta^{13}C_{leaf}$ and WUE_{plant} had a strong negative correlation within the allele classes defined by $\delta^{13}C_{leaf}$.

Both WUE_{plant} and WUE_{fit} are driven by a balance between the intrinsic relationship between carbon assimilation and water lost via stomates (TE_i) and other whole plant processes such as ϕ_c, ϕ_w, and r (when only aboveground biomass is measured). Hence, the relationship between $\delta^{13}C_{leaf}$ and WUE_{plant} is only apparent if TE_i has a strong influence on WUE_{plant}. Nevertheless, $\delta^{13}C_{leaf}$ can be used to detect variation in TE_i that is not apparent from measuring WUE_{plant} only. Therefore, this study advances our understanding of WUE_{plant}, TE_i, and $\delta^{13}C_{leaf}$ in a C_4 species. Furthermore, it illustrates that $\delta^{13}C_{leaf}$ has potential to be used in screening for TE_i in marker-assisted C_4 plant breeding and to better understand the genetic controls of WUE_{plant} and its components. Additional work is needed to explore the use of $\delta^{13}C_{leaf}$ in other C_4 species and under field settings to better understand the complex interaction of traits and causal genes that influence WUE_{plant}, TE_i, and $\delta^{13}C_{leaf}$.

Methods

Plant material and growth conditions

An interspecific *Setaria* F7 recombinant inbred line (RIL) population comprised of 189 genotypes was previously generated through a cross between the wild-type green foxtail *S. viridis* accession, A10, and the domesticated *S. italica* foxtail millet accession, B100 (60, 61, 71). Seeds from this population were sowed in 10 cm diameter white pots pre-filled with ~470 cm^3 of Metro-Mix 360 soil (Hummert, USA) and 0.5 g of Osmocote Classic 14-14-14 fertilizer (Everris, USA) and placed on the Bellwether Phenotyping System using a random block design. Two to three replicates per genotype, including the A10 and B10 parental accessions, per treatment (1138 individuals) were grown for 25 days with a photoperiod of 16 h light / 8 h night, light intensity of 500 $\mu mol/m^2/s$, a temperature regime of 31°C day/21°C night and relative humidity was maintained between 40 – 80 %. Plants were watered 2-3 times per day to maintain plants at 100 % pot capacity (PC) in the well-watered treatment and at 40% PC in the water-limited treatment as determined by Fahlgren, *et al.* (41). Prescribed soil water content across both treatment blocks was achieved by 15 days after planting. Additional detail on the experimental design and plant growth can be found in Feldman, *et al.* (42), Feldman, *et al.* (66).

Measurements of biomass and transpiration

The volume of water transpired by individual plants at each pot weighing was calculated as the difference between the measured pot weight and the weight of the pre-filled pot at pot capacity (100% PC for the well-watered treatment) or the difference between current pot weight and the weight measurement on the previous day if no water was added. The pots were watered to return the pot weight to the pre-set pot weight, which maintains the gravimetric water content at either 100 % or 40 % PC for the well-watered or water-limited treatment blocks, respectively. Initially all seedlings were watered to pot capacity for the first two days on the Bellwether system. After two days, the potting medium in the water-limited treatment was allowed to dry down to 40 % of PC, then maintained at the water content level of 40 %.

Plants were imaged every other day to measure side view area, which was used to calculate fresh biomass (41, 42). The relationship between side view area and fresh biomass was developed based on the final aboveground biomass and the last imaging before harvesting. The sensitivity of the measurements limited reliable measurements of

plant size and transpiration from day 17 to 33 when plants were not too small for image analysis and measurements of transpiration (42). Water use efficiency (WUE_{ratio}) was calculated as the ratio of aboveground biomass and total water transpired. Instead of comparing $\delta^{13}C_{leaf}$ to each day, the midpoint of the experiment was chosen (day 25) to conduct the analysis. The plants on day 25 were in vegetative phase and growing rapidly, typical of when physiological and gas exchange measurements would be made. Additionally, day 25 was representative in terms of QTL and other analyses conducted throughout the experiment (42).

In this population, plant size and cumulative whole-plant transpiration were strongly correlated (42), so an ordinary least squares linear regression was used within treatment blocks to model this relationship (Fig. S3). The within treatment model was used to predict the accumulation of fresh biomass based on the amount of water transpired (WUE_{fit}). The residuals surrounding WUE_{fit} (WUE_{res}) represent the fresh biomass of individual plants not explained by WUE_{fit} (42).

Leaf carbon stable isotopic composition ($\delta^{13}C_{leaf}$)

The youngest, fully expanded leaf was collected during the final harvest at the end of the experiment (day 34) and dried at 55 °C for three days. Once the leaves were dried, 8-12 discs, having a total leaf area of 0.47 – 0.71 cm^2, were sampled from each leaf and placed in tin capsules for stable isotopic analysis. A comparison of $\delta^{13}C_{leaf}$ from sampling leaf discs to grinding and sampling an aliquot of the completely homogenized powered leaf tissue was made on a subset of 47 leaves. The slope of $\delta^{13}C_{leaf}$ from the punches regressed against $\delta^{13}C_{leaf}$ from the ground leaf tissue was 0.93 ± 0.03 (R^2 = 0.96; Fig. S4) and the mean difference between methods was 0.06 ± 0.04 ‰, which was similar to the IRMS precision and significantly less than the sample standard deviation of 0.5 ‰. Considering the similarity between sampling methods, all leaves were sampled using the more rapid hole punching method.

Leaf tissue was converted to CO_2 with an elemental analyzer (ECS 4010, Costech Analytical, Valencia, CA) and analyzed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, ThermoFinnigan, Bremen; (72, 73). The Santrock correction was used by the IRMS software to correct for ^{17}O (74). Final δ values were the mean of 5 sample peaks calibrated to the international standards NBS 19, RM 8542, and IAEA-CO-9 to calculate $\delta^{13}C$ relative to Vienna Peedee belemite (V-PDB). Quality control

351 standards were also included to determine the correction quality. Overall standard
352 deviation for $\delta^{13}C$ values was 0.07 ‰.

353 The stable isotope composition of carbon ($\delta^{13}C_{leaf}$) was reported in δ notation in
354 parts per thousand (‰),

$$\delta = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \qquad \text{Equation 1}$$

355 where R_{sample} and $R_{standard}$ is the molar ratios of heavy to light isotope ($^{13}C/^{12}C$) of the
356 sample and international standard, respectively. The international standard used for
357 oxygen was Vienna- PeeDee Belemite (VPDB).

358 *Statistical analysis*

359 Statistical analyses were conducted in R version 3.4.0 (R Team 75), using car
360 (version 2.0-26) packages for statistical tests and lmodel2 (version 1.7-2) package for
361 Model II regression analysis (standard major axis). Model II regressions were calculated
362 because neither variable was controlled, both varied naturally with their own associated
363 error, and the physical units of both variables were not the same. Homogeneity was
364 tested based on plotting predicted fit versus residuals. Using the extRemes package
365 (version 2.0-8), normality was tested by plotting residuals on quantiles-quantiles plots.
366 Within treatment comparisons were made on each trait using a two-factor analysis of
367 variance (ANOVA) where the factors were treatment and genotype. Slope of the
368 relationship between fresh biomass and transpiration was calculated with Model II
369 linear regression and determined to be different using analysis of co-variance
370 (ANCOVA). Methods used in QTL analysis were explained in Feldman, *et al.* (42),
371 Feldman, *et al.* (66).

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375

Appendix I: Glossary of terms

Term	Definition
$\Delta^{13}C$	Photosynthetic carbon discrimination ($\delta^{13}C_{ambient}$ - $\delta^{13}C_{leaf}$)
$\delta^{13}C_{leaf}$	Leaf carbon isotopic composition (‰)
$\delta^{13}C_{ambient}$	Isotopic composition of ambient CO_2 (‰)
WUE_{ag}	Agricultural water use efficiency (crop yield/water applied to crop)
WUE_{plant}	Total biomass/water transpired
$WUE_{biomass}$	Theoretical ratio of aboveground biomass and water transpired
WUE_{ratio}	Measured ratio of aboveground biomass and water transpired
WUE_{fit}	Linear model fit of biomass regressed on transpiration
WUE_{res}	Residuals of the linear model between biomass and transpiration
TE_i	Intrinsic transpiration efficiency (A_{net}/g_s)
HI	Harvestable index
C_i/C_a	Intercellular to ambient CO_2 concentration
g_s	Stomatal conductance
A_{net}	Net photosynthetic rate
T	Transpiration rate
ET	Evapotranspiration
v	Evaporative demand; ($e_i - e_a$)
e_i - e_a	Water vapor molar difference between intercellular and ambient air at leaf temperature
ϕ_w	Proportion of water used by plant that is unproductive water loss (*e.g.* nighttime and cuticular transpiration)
ϕ_c	Proportion of fixed carbon lost through respiration
r	Proportion of biomass
a	Fractionation during diffusion of CO_2 in air through stomata (4.4 ‰)
b_3	Fractionation by Rubisco (30 ‰)
b_4	Fractionation of PEP carboxylation and isotopic equilibrium during dissolution and hydration of CO_2 (–5.2 ‰ at a leaf temperature of 30 °C)
s	Fractionation during the CO_2 leakage from the bundle sheath cells (1.8 ‰)
ϕ	Leakiness of CO_2 from the bundle sheath
H^2	Broad sense heritability

Appendix II. Theory

Agricultural water use efficiency (WUE_{ag}) can be defined as the crop yield per unit water supplied to the crop system. Therefore, yield relative to water use can be calculated as a function of evapotranspiration (ET), the proportion of ET that is transpired (T/ET), WUE_{plant}, and the harvest index (harvested proportion of biomass; Equation 1; 2, 15) as

$$Yield = ET\ x\ \frac{T}{ET}\ x\ WUE_{plant}\ x\ HI. \qquad \text{Equation S1}$$

Where WUE_{plant} relates to net CO_2 assimilation rates (A_{net}) relative to transpiration rate (T) at the whole plant level, and accounts for the proportion of fixed carbon lost by whole plant respiration (ϕ_c) and the proportion of "unproductive" water loss (ϕ_w) such as nighttime transpiration or cuticular evaporation (Equation 2; 11, 76). The relationship of these parameters to WUE_{plant} can be defined as

$$WUE_{plant} = \frac{A_{\text{net}}(1-\phi_c)}{T(1+\ \phi_w)} \qquad \text{Equation S2}$$

where A_{net} and T are related through stomatal conductance (g_s) as

$$A_{\text{net}} = g_{sCO2}(C_a - C_i) \qquad \text{Equation S3}$$

and

$$T = g_{sH2O}(e_i - e_a). \qquad \text{Equation S4}$$

The parameter (e_i - e_a) is the water vapor molar difference between intercellular and ambient air at leaf temperature, (C_a - C_i) is the CO_2 molar difference between intercellular and ambient CO_2, and g_{sCO2} and g_{sH2O} are the conductance values for CO_2 and H_2O, respectively (10-12). Substituting Eqs 3 and 4 into Eq 2 gives

$$WUE_{plant} = \frac{g_{sCO2}(C_a - C_i)(1-\phi_c)}{g_{sH2O}(e_i - e_a)(1+\ \phi_w)} = \frac{C_a\left(1-\frac{C_i}{C_a}\right)(1-\phi_c)}{1.6v(1+\ \phi_w)} \qquad \text{Equation S5}$$

where v is the evaporative demand (e_i-e_a), and the ratio of diffusivities of H_2O and CO_2 in air is 1.6. The molar ratio of intercellular to ambient CO_2 (C_i/C_a) influences WUE_{plant} because it represents the relative drawdown of intercellular CO_2 (C_i) by photosynthesis and the conductance of CO_2 into the leaf and the conductance of water vapor out the leaf via the stomata. Intrinsic TE (A_{net}/g_s; TE_i) is equal to the CO_2 gradient from ambient to intercellular spaces (C_i - C_a), which can be rewritten as $TE_i = C_a(1-C_i/C_a)$.

Therefore, Eq 5 can be simplified as a function of TE_i as

$$WUE_{plant} = \frac{TE_i(1-\phi_c)}{1.6v(1+\ \phi_w)} \qquad \text{Equation S6}$$

When WUE is calculated with respect to aboveground biomass ($WUE_{biomass}$) as is the case with a bioenergy crop, biomass yield excludes the root fraction (r) and $WUE_{biomass}$ can be defined as

$$WUE_{biomass} = \frac{TE_i(1-\phi_c)(1-r)}{1.6v(1+\ \phi_w)} \qquad \text{Equation S7}$$

The relationship between $\delta^{13}C_{leaf}$ and TE_i is based on 1) variation in $\delta^{13}C_{leaf}$ (‰) of plants grown in the same atmospheric conditions is primarily controlled by leaf CO_2 isotope discrimination ($\Delta^{13}C$), 2) $\Delta^{13}C$ is influenced by changes in C_i/C_a and 3) C_i/C_a, as stated above, is affected by the interrelationship A_{net} and g_s. Therefore, TE_i (A_{net}/g_s) is related to C_i/C_a and, in turn, $\Delta^{13}C$ (10, 27).

Finally, leaf carbon composition ($\delta^{13}C$) is related to $\Delta^{13}C$ as

$$\Delta^{13}C = \frac{\delta^{13}C_{ambient} - \delta^{13}C_{leaf}}{1+\delta^{13}C_{leaf}/1000} \qquad \text{Equation S8}$$

where, $\delta^{13}C_{ambient}$ is the CO_2 isotopic signature of the CO_2 in the air surrounding the leaf, and $\delta^{13}C_{leaf}$ is the leaf carbon isotopic composition (20). In C_4 species, $\Delta^{13}C$ is primarily determined by fractionations associated with CO_2 carboxylation and diffusion, the ratio of bundle sheath CO_2 leak rate to PEP carboxylase rate (leakiness; ϕ), and C_i/C_a (Equation 9). Leakiness (ϕ) determines the slope of the relationship between $\Delta^{13}C$ and C_i/C_a and has been shown to be relatively constant in many C_4 species across light intensities, temperatures, and CO_2 partial pressures (77-80). Based on this mathematical

relationship, if ϕ is less than 0.37, then $\Delta^{13}C$ increases as C_i/C_a decreases, which corresponds with increasing $\delta^{13}C_{leaf}$. If ϕ is greater than 0.37, then the relationship reverses where $\Delta^{13}C$ increases with C_i/C_a. In *Setaria*, ϕ has been found to be less than 0.37, so C_i/C_a is expected to form a negative relationship with $\Delta^{13}C$ and positive relationship with $\delta^{13}C_{leaf}$ (Ellsworth et al. unpublished; 81). The relationship of $\Delta^{13}C$ and C_i/Ca can be defined by the simplified relationship as originally described by Farquhar (1984) as

$$\Delta^{13}C = a + (b_4 + \phi(b_3 - s) - a)\frac{C_i}{C_a} \qquad \text{Equation S9}$$

where a is the fractionation during diffusion of CO_2 in air through stomata (4.4 ‰), b_4 is the fractionations of PEP carboxylation and the preceding isotopic equilibrium during dissolution and hydration of CO_2 (-5.7 ‰ at a leaf temperature of 25 °C) as described in (82, 83), b_3 is the Rubisco fractionation (29 ‰), and s is the fractionation during the leakage of CO_2 out of the bundle sheath cells (1.8 ‰) (27, 82).

Table 1: Analysis of variance of traits. Means ± SD of fresh biomass, transpiration, WUE_{ratio}, WUE_{fit}, and WUE_{res} were determined on day 25. $\delta^{13}C_{leaf}$ was collected at the end of the experiment on day 34.

Trait	Treatment block		Treatment		Genotype		Treatment x Genotype	
	Well-watered	Water-limited	$F_{ddf,ndf}$	P	$F_{ddf,ndf}$	P	$F_{ddf,ndf}$	P
Fresh biomass (g)	14.87 ± 6.78	5.29 ± 2.04	$4248_{1,744}$	<0.0001	$14.83_{188,744}$	<0.0001	$6.11_{182,744}$	<0.0001
Transpiration (ml)	765.4 ± 60.1	268.7 ± 193.4	$8395_{1,764}$	<0.0001	$7.432_{189,764}$	<0.0001	$3.321_{183,764}$	<0.0001
WUE_{ratio} (g/L)	19.7 ± 5.0	24.5 ± 5.0	$515.8_{1,744}$	<0.0001	$6.613_{188,744}$	<0.0001	$1.063_{182,744}$	0.29
WUE_{fit} (g)	14.87 ± 6.12	5.29 ± 1.54	$3299_{1,744}$	<0.0001	$7.441_{188,744}$	<0.0001	$3.942_{182,744}$	<0.0001
WUE_{res} (g)	-1.85 ± 2.93	-1.85 ± 1.34	$0_{1,744}$	1.00	$2.785_{188,744}$	<0.0001	$0.97_{182,744}$	0.59
$\delta^{13}C_{leaf}$	-13.50 ± 0.50	-14.33 ± 0.55	$569_{1,351}$	<0.0001	$2.054_{184,351}$	<0.0001	$1.003_{175,351}$	0.49

Table 2: Broad-sense heritability (H^2) and proportional variance of traits on day 25 after sowing. $\delta^{13}C_{leaf}$ was collected at the end of the experiment.

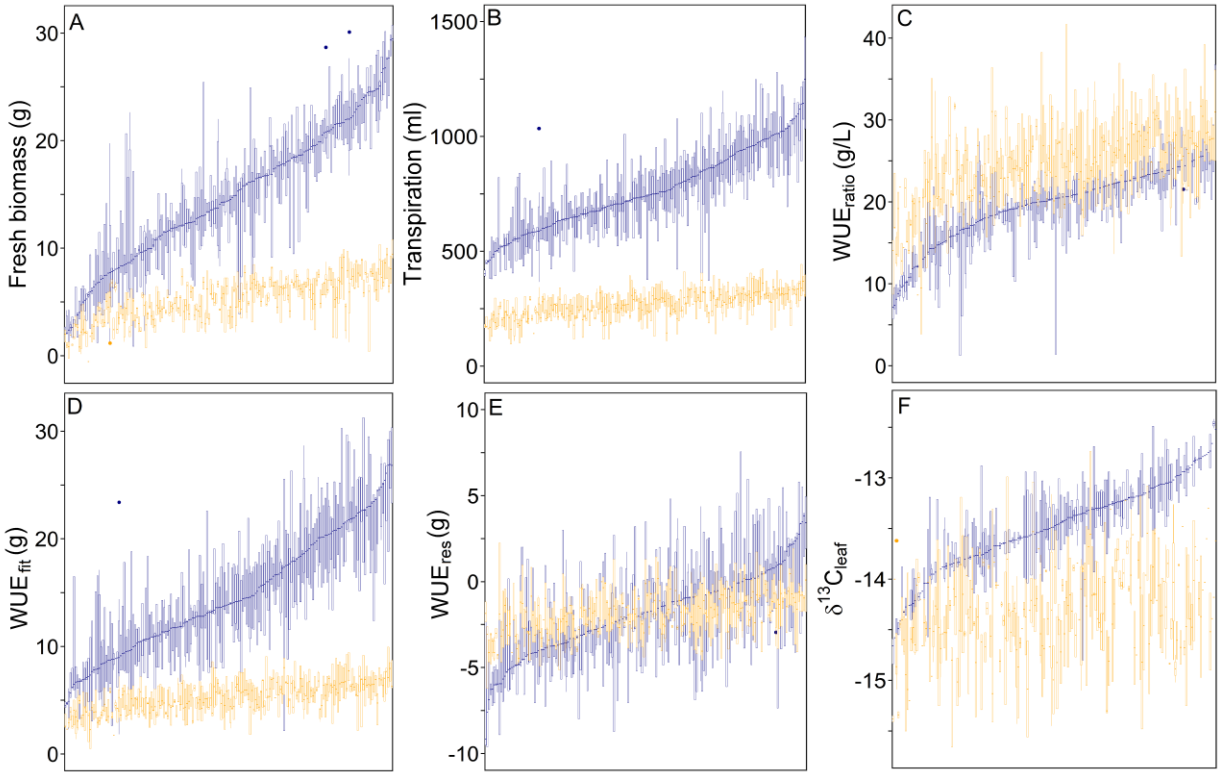
Trait	**Proportional variance**				**H^2**	
	Genotype	**Treatment**	**G x Treatment**	**Both treatments**	**Well-watered treatment**	**Water-limited treatment**
Fresh biomass	0.13	0.64	0.14	0.60	0.77	0.65
Transpiration	0.04	0.86	0.04	0.56	0.61	0.34
WUE_{ratio}	0.34	0.32	0.01	0.83	0.62	0.37
WUE_{fit}	0.07	0.69	0.11	0.48	0.63	0.33
WUE_{res}	0.24	0.0	0.0	0.62	0.23	0.27
$\delta^{13}C_{leaf}$	0.09	0.55	0.01	0.45	0.49	0.04

Table 3. QTL found across all traits in both treatments. Filled cells represent QTL, and the number in the cell is the proportion of additive variance explaince (%) by the QTL, which can have a positive or negative effect on the trait.

Trait	**Treatment**	**Genomic position of QTL**							
		2@96	**3@48**	**5@109**	**6@61**	**7@51**	**7@99**	**9@34**	**9@126**
Fresh biomass	well-watered	15.47	5.5	-6.22	4.8		-8.24	11.88	
	water-limited	10.3		-8.52			-5.19	20.37	9.5
Transpiration	well-watered	13.45		-9.92	4.8	-5.14	-10.54	16.49	
	water-limited	8.15					-11.54	21.84	
WUE_{fit}	well-watered	13.45		-9.92		-5.14	-10.53	16.49	
	water-limited	8.15					-11.54	21.84	
$\delta^{13}C$	well-watered					-6.49	-8.2	14.52	
	water-limited								
WUE_{ratio}	well-watered	20.27		-10.67				6.45	
	water-limited	13.56		-11.77				9.15	7.50
WUE_{res}	well-watered	7.96		-13.55					
	water-limited	11.01		-22.72					

Table 4: $\delta^{13}C_{leaf}$ and the regression slope of the relationship between fresh biomass and transpiration at the allele class level. The slopes are Model II regression (standard major axis). The allele class 'ABA' was not present in this RIL population. These slopes ± SEM are from the relationship found in figure 3A and are also plotted against $\delta^{13}C_{leaf}$ ± SEM in figure 3B.

	Well-watered treatment				**Water-limited treatment**			
Allele class	**Slope**	**R^2**	***P***	**$\delta^{13}C_{leaf}$**	**Slope**	**R^2**	***P***	**$\delta^{13}C_{leaf}$**
AAA	36.8 ± 3.7	0.85	< 0.0001	-13.7 ± 0.08	45.4 ± 3.9	0.87	< 0.0001	-14.36 ± 0.07
AAB	35.1 ± 2.3	0.78	< 0.0001	-13.15 ± 0.04	35.7 ± 3.2	0.59	< 0.0001	-14.28 ± 0.05
ABA	Not present in RIL population							
ABB	38.4 ± 15.9	0.49	0.19	-13.8 ± 0.07	31.1 ± 16.5	0.16	0.51	-14.24 ± 0.16
BBB	61.1 ± 6.6	0.91	< 0.0001	-13.91 ± 0.07	53.1 ± 9.2	0. 59	0.009	-14.42 ± .13
BBA	56.7 ± 11.2	0.81	0.006	-14.0 ± 0.09	62.7 ± 8.8	0.90	0.001	-14.60 ± 0.10
BAB	35.1 ± 1.7	0.86	< 0.0001	-13.44 ± 0.04	39.6 ± 2.5	0.76	< 0.0001	-14.23 ± 0.05
BAA	47.9 ± 4.0	0.86	< 0.0001	-13.79 ± 0.09	42.6 ± 7.8	0.32	0.006	-14.43 ± 0.08



Correlation with $\delta^{13}C_{leaf}$ (r)					
Treatment	Fresh biomass	Transpiration	WUE_{ratio}	WUE_{fit}	WUE_{res}
Well-watered	0.30	0.33	0.20	0.33	-0.03
Water-limited	-0.05	-0.05	-0.06	-0.05	-0.01
Both treatments	0.51	0.60	-0.19	0.55	-0.12

Figure 1. Ordered boxplots of fresh biomass (A), transpiration (B), WUE_{ratio} (C), WUE_{fit} (D), WUE_{res} (E), and $\delta^{13}C_{leaf}$ (F). All traits were measured on day 25 at peak growth, except $\delta^{13}C_{leaf}$, which $\delta^{13}C_{leaf}$ measured on leaves collected at the end of the experiment on day 34. Treatment effect was significant for all traits except WUE_{res}. The table below shows the correlation coefficients of each trait with $\delta^{13}C_{leaf}$.

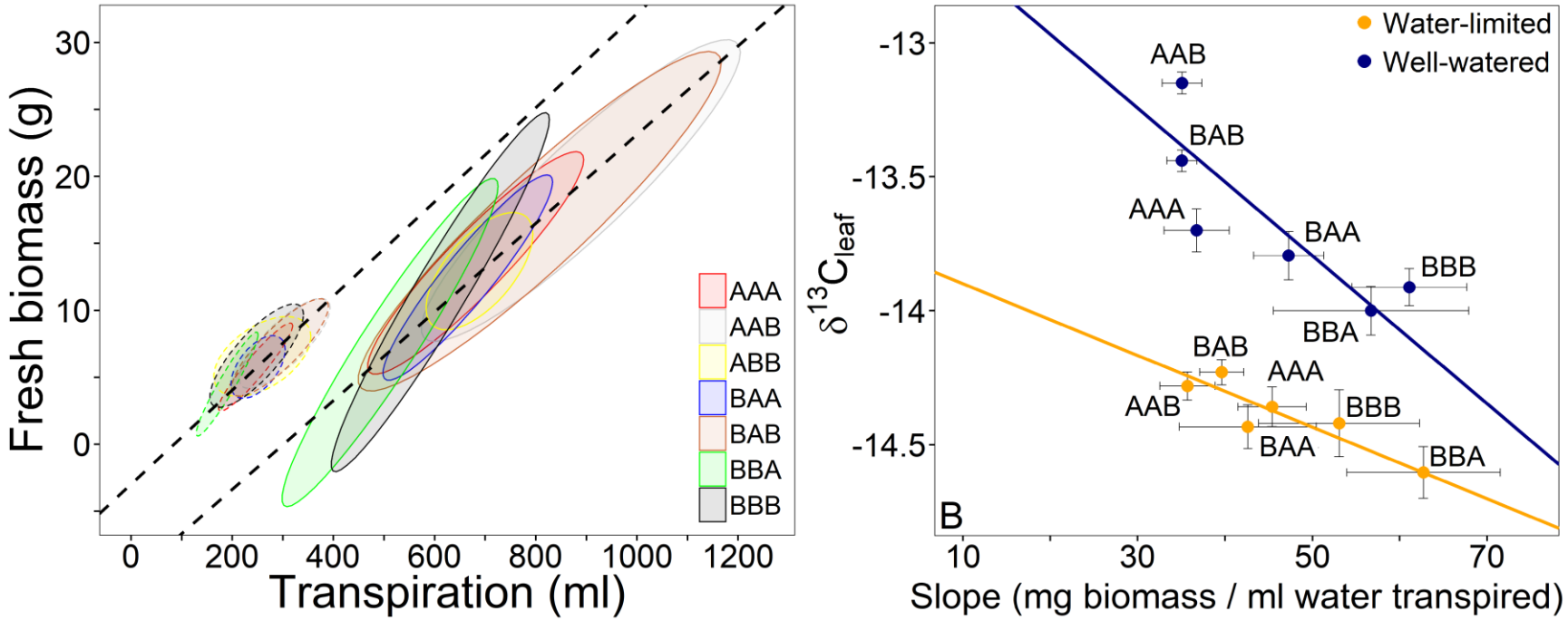


Figure 2. The effect of allele class on fresh biomass, transpiration and $\delta^{13}C_{leaf}$. In panel A, QTL 7@51, 7@99, and 9@34 were combined to produce seven alelle classes where the first letter represents the allele at QTL 7@51, the second letter represents the allele at QTL 7@99, and the third letter represents the allele at QTL 9@34. The letter 'A' represents the allele from the A10 parental accession (*Setaria viridis*), and 'B' represents the allele from the B100 parental accession (*Setaria italica*). Ellipses represent 95 % confidence intervals for the relationship of fresh biomass and transpiration, and the slope of this relationship for each allele class was significant, except for allele class 'ABB' ($P < 0.0001$). In panel B, $\delta^{13}C_{leaf}$ ± SEM is regressed against the slope of relationship ± SE in panel A, excluding the non-significant slope for 'ABB'. The slope is the water use efficiency for an entire allele class. The regression for $\delta^{13}C_{leaf}$ versus slope was significant in the well-watered ($\delta^{13}C_{leaf}$ = -0.027 slope – 12.48; $R^2 = 0.71$; $P < 0.05$) and in the water-limited treatments ($\delta^{13}C_{leaf}$ = -0.012 slope – 13.82; $R^2 = 0.80$, $P < 0.05$).

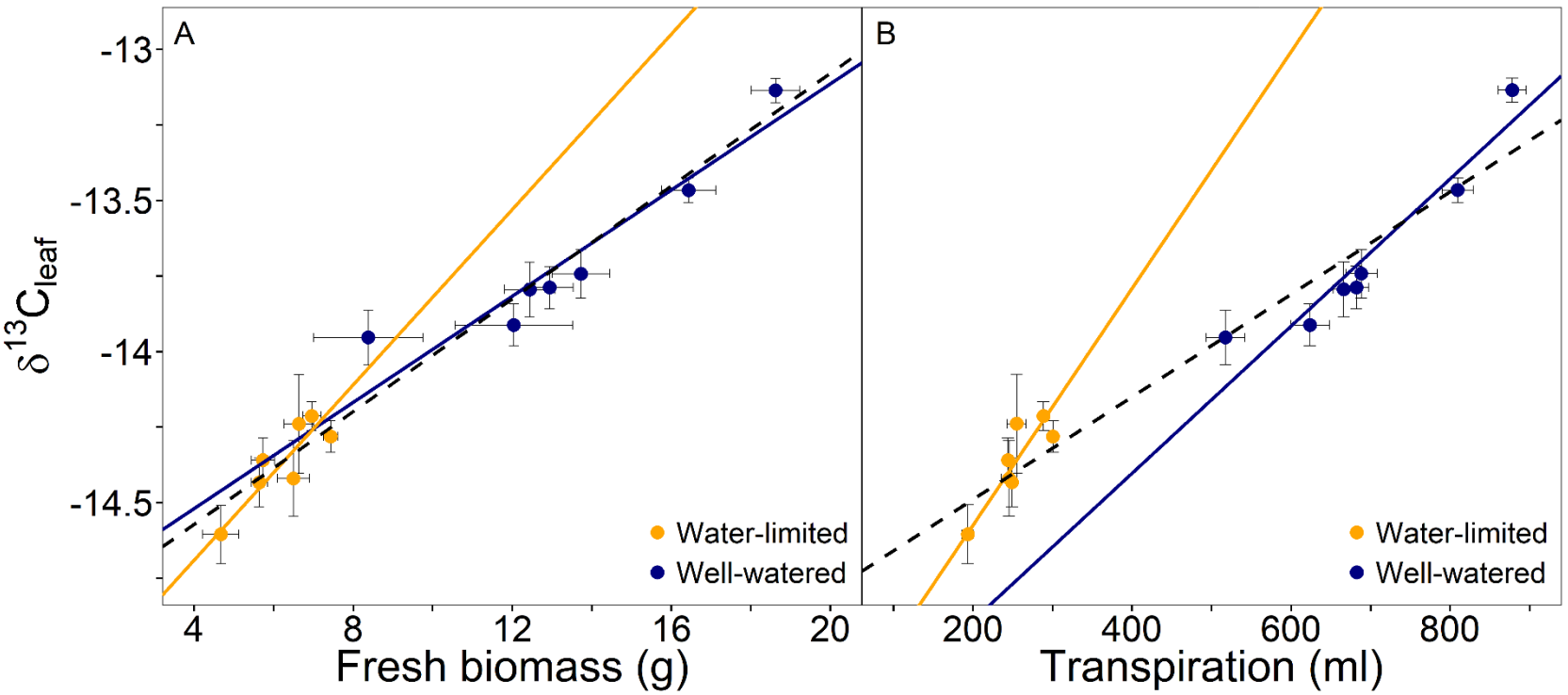


Figure 3. The effect of allele class on the relationship of fresh biomass and transpiration with $\delta^{13}C_{leaf}$. Like in Fig. 2, QTL 7@51, 7@99 and 9@34 were combined to produce seven alelle classes. In panel A, the mean $\delta^{13}C_{leaf}$ was regressed against fresh biomass for both treatments separately and both combined ($\delta^{13}C_{leaf}$ = 0.088 fresh biomass – 14.87; R^2 = 0.88; P = 0.002 for well-watered; $\delta^{13}C_{leaf}$ = 0.145 fresh biomass – 15.14; R^2 = 0.75; P = 0.01 for water-limited; $\delta^{13}C_{leaf}$ = 0.093 fresh biomass – 14.94; R^2 = 0.95; P < 0.0001 for both treatments). In panel B, $\delta^{13}C_{leaf}$ is regressed against transpiration for both treatments and both treatments combined ($\delta^{13}C_{leaf}$ = 0.0024 transpiration – 15.38; R^2 = 0.90; P = 0.001 for well-watered; $\delta^{13}C_{leaf}$ = 0.0039 transpiration – 15.36; R^2 = 0.75; P = 0.01 for water-limited; $\delta^{13}C_{leaf}$ = 0.0017 transpiration – 14.83; R^2 = 0.93; P < 0.0001 for both treatments). Regression of both treatments together is identified by black, dashed line.

Supplemental Figures

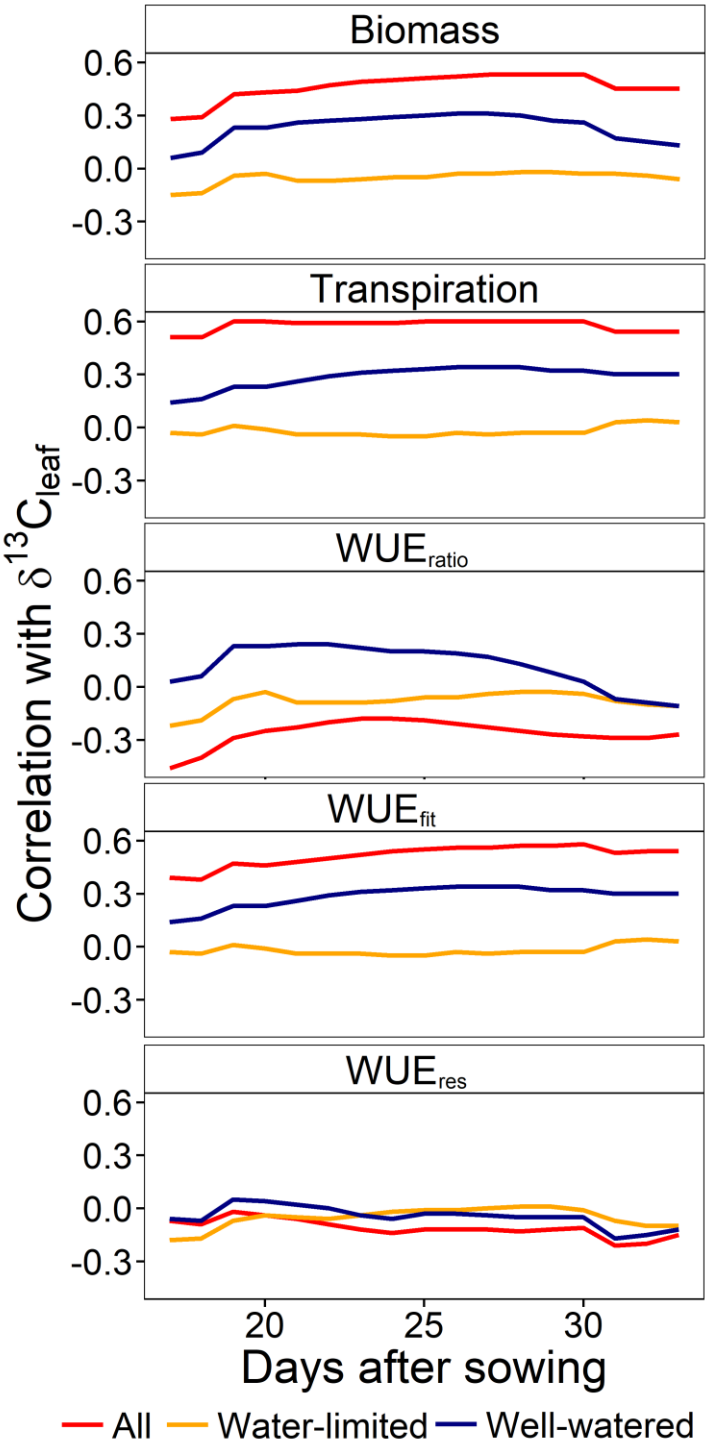


Figure S1. Correlation of fresh biomass, transpiration, WUE_{ratio}, WUE_{fit}, and WUE_{res} with $\delta^{13}C_{leaf}$ through the course of the experiment. Fresh biomass, transpiration, WUE_{fit}, and WUE_{res} were from Feldman, *et al.* (42).

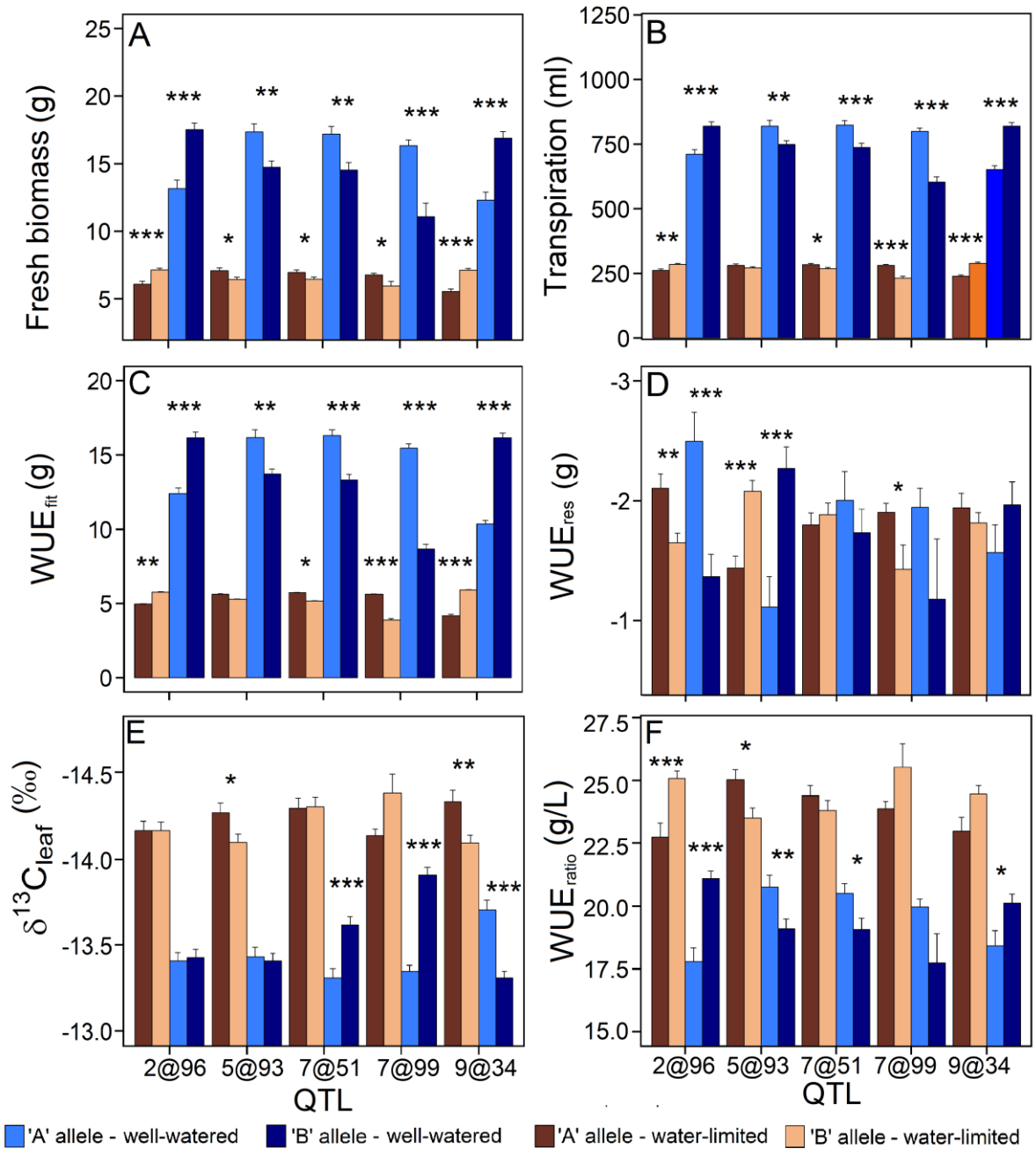


Figure S2. Effect of alleles of the five principal QTL identified for fresh biomass (A), transpiration (B), WUE_{fit} (C), WUE_{res} (D), and $\delta^{13}C_{leaf}$ (E), and WUE_{ratio} (F). 'A' represents the allele from the A10 parental line (*Setaria viridis*), and 'B' represents the allele from the B100 parental line (*Setaria italica*). Level of significance is denoted as the following: *, **, *** represent $P < 0.05$, 0.01, 0.0001, respectively.

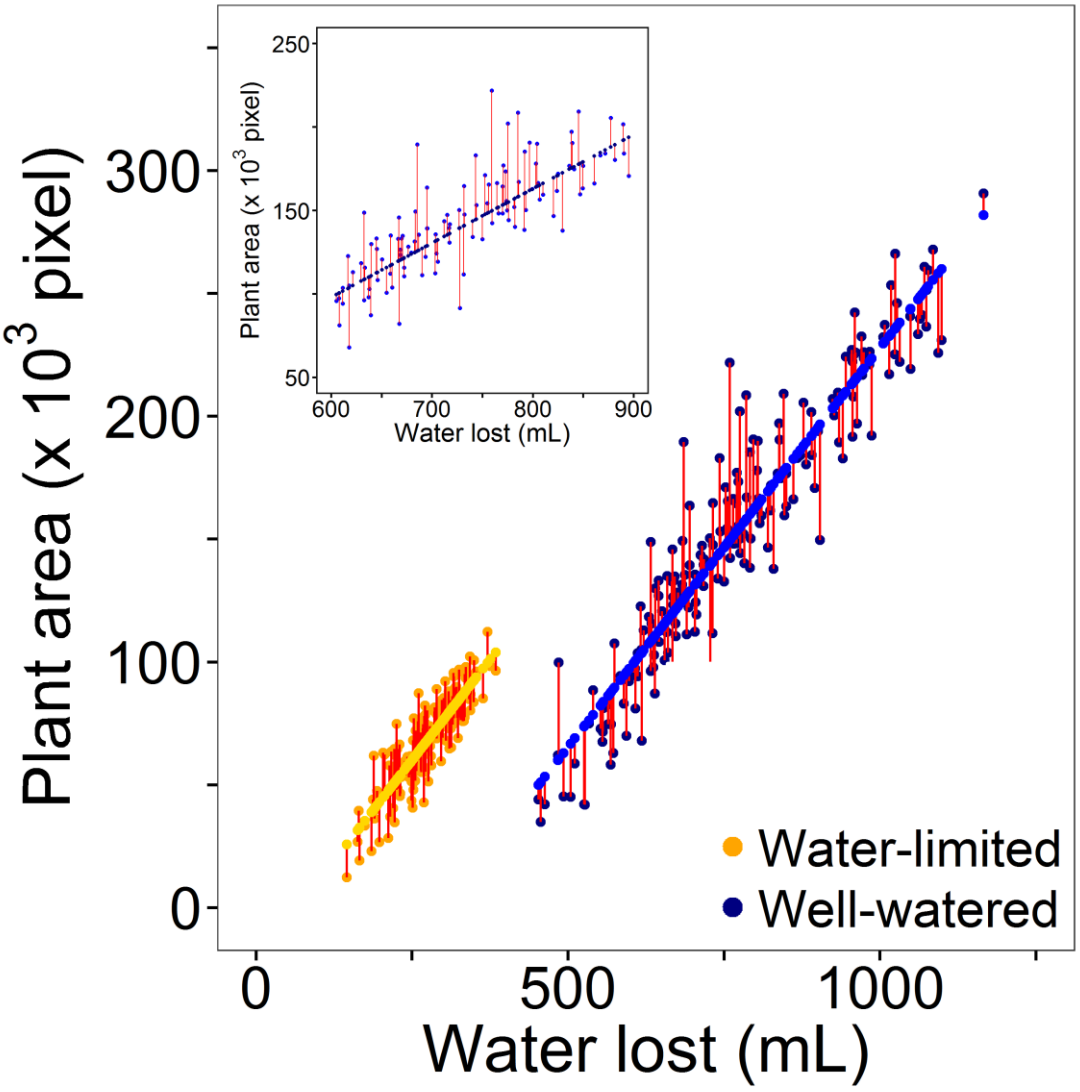


Figure S3. Method of measuring the residuals of an ordinary least squares (OLS) linear regression for plant size versus whole-plant transpiration on day 25. Blue and yellow points represent the model fit (WUE_{fit}) in each treatment. Red vertical lines connecting points to the model fit (WUE_{res}) represent residuals. Inset zooms in to the range from 600-900 ml of cumulative transpiration for the well-watered treatment only.

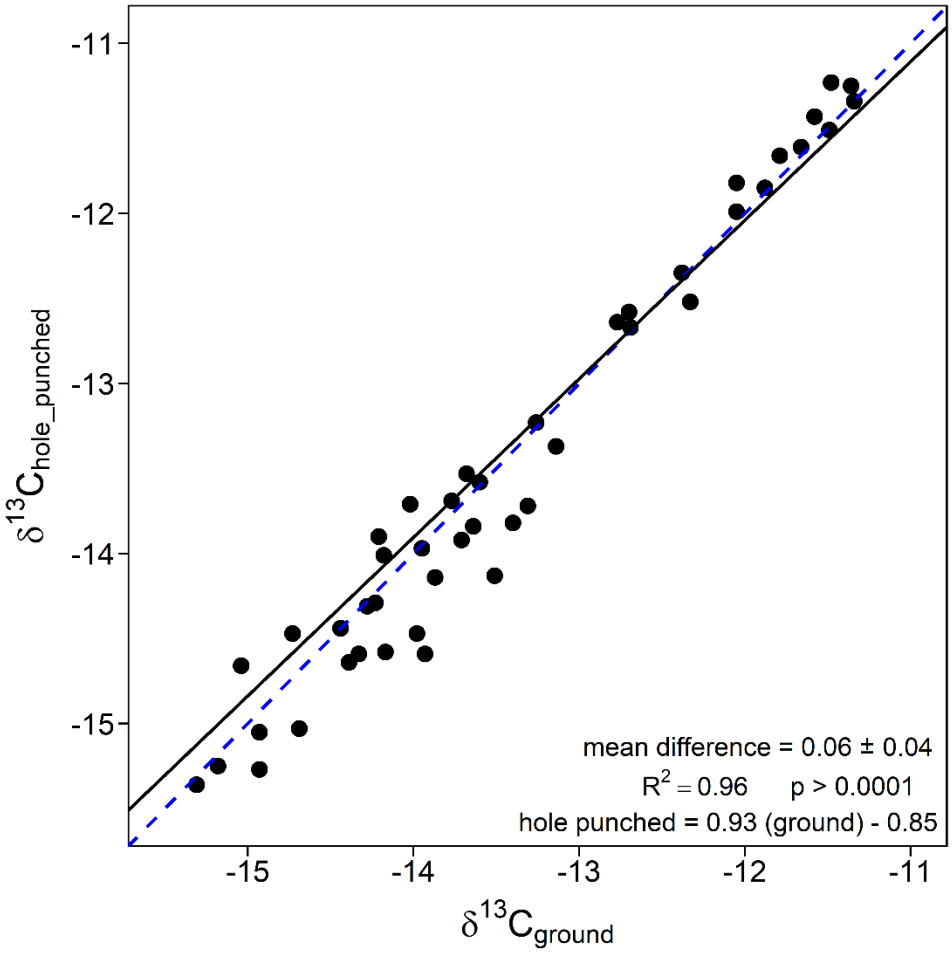


Figure S4. Comparison of the two sampling methods for $\delta^{13}C_{leaf}$. Principal method used to sample for $\delta^{13}C_{leaf}$ was hole punching, which provided similar values to grinding the entire leaf and weighing out a subsample. Solid, black line represents the regression line. The dashed, blue represents 1:1 line.

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