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Fast stomatal responses require anisohydric stomatal behaviour and lower water use efficiency in sorghum

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Abstract: Intrinsic water use efficiency (iWUE), the ratio of leaf carbon assimilation (A_n) to stomatal conductance to water vapour (g_s), is an important crop trait. The effect of environmental fluctuations, such as light transitions, on iWUE are mediated via the stomatal kinetic responses to these fluctuations. We screened for variation in stomatal kinetic response to changes in light intensity in a sorghum population containing different haplotypes, selected based on variation in aquaporin (AQP) alleles. We assessed the role of stomatal anatomy in determining iWUE. Furthermore, we grew plants under water stress to reveal the trade-offs between water use and conservation underlined by stomatal kinetics and behavior. The results showed that iWUE measured from steady state A_n/g_s and iWUE calculated from A_n and g_s responses to transient light change (dynamic iWUE) correlated, propelled by prolonged stomatal opening time (k_{open}) but independent of stomatal closing time (k_{close}). Stomatal size and density played a minor role in determining those responses but still might have an indirect effect. Leaf width correlated instead with faster stomatal opening and increased anatomical conductance. "Faster" stomata were significantly determined by decreased regulation of leaf water potential (increased anisohydry) because they had reduced sensitivity to changes in leaf water status, while "slower" stomata were characterized by water conservation and heightened sensitivity to changes in leaf water potential. We found those two sets of behaviors can be distinguished by an AQP-associated haplotype (SbTIP3.2), opening the door for newer genetic determinants of iWUE that can incorporate possible trade-offs.

Keywords: Water use efficiency; Stomatal kinetics; Sorghum; Stomatal sensitivity to drought; Stomatal anatomy

1. Introduction

Anthropogenic activity fueling climate change has increased drought frequency and temperature extremes [1, 2]. Combined with increased worldwide population and scarcity of freshwater resources, the agricultural sector is now prioritizing water productivity, with an emphasis on achieving higher crop yields with limited water resources [3]. This water productivity trait is termed water use efficiency (WUE). Plant physiologists define WUE, at the leaf level, as the

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ratio of the rates of photosynthetic CO_2 assimilation (A_n) to transpiration rate (E) [4]. However, rates of transpiration are controlled by stomatal conductance (g_s) and other environmental variables, thus for breeding purposes a more solid genetic indicator of water loss capacity is g_s, hence the usage of leaf intrinsic WUE (iWUE, A_n/g_s) [5]. Improving iWUE in C_4 crops like sorghum is now a priority as it is cultivated in areas expected to be impacted harder by climate change [6, 7]. Sorghum is known as a drought and heat tolerant crop, making it a good candidate to contain diverse drought tolerant traits within its germplasm [8].

Steady-state iWUE (iWUE$_{SS}$) is determined by the final size of the stomatal aperture that determines g_s [9]. However, under changing environmental cues such as light, the aperture requires time to reach its final size, and the time taken is often an order of magnitude higher than the time required for A_n to respond to changes in light intensity [10]. This discrepancy has a significant influence on iWUE over the day [11]. In a fluctuating environment such as agricultural fields, light intensity is one of the most variable environmental conditions [12]. The more time ("slower") g_s takes to increase in response to high light intensities restricts CO_2 uptake, but a slower g_s decline in response to lower light intensity causes needless water loss. As a result, rapid g_s responses are thought to maximize both A_n and iWUE by rapidly reaching steady-state values when light levels change, such as during sunflecks [13]. Hence, optimizing iWUE during transition to and from different light levels (dynamic iWUE, iWUE$_{dyn}$) can be a strong indicator of temporally integrated leaf WUE. However, higher iWUE$_{dyn}$ can be the result of slower stomatal opening, which limits CO_2 uptake but reduces water loss overall to increase iWUE due to the high dependence of iWUE on g_s rather than A_n [14]. Theoretically, iWUE$_{dyn}$ can increase via faster stomatal opening, reducing the asymmetry between the A_n and g_s response, and increasing iWUE without sacrificing CO_2 uptake and photosynthesis [14]. This possible trade-off between iWUE and productivity can be a significant oversight of breeding for higher WUE [15], and our starting aim was to explore this trade-off in dynamic conditions (light transitions) in a large sorghum population.

Stomatal anatomy, specifically size (SS) and density (SD), determines maximum g_s and influences stomatal kinetics [10]. Smaller guard cells have a greater cell surface area to volume ratio, which may allow faster ion fluxes, guard cell turgor changes, and g_s responsiveness [16]. This led to the hypothesis that smaller stomata take less time ("faster") to reach steady-state and are more water use efficient [11, 17]. However, these correlations between size and kinetics are not always consistent, especially in crops [11, 18–20], limiting the potential of selecting for certain stomatal traits to improve iWUE. Concurrent with stomatal anatomy, leaf width (LW) has been shown to influence stomatal anatomy and impact g_s in sorghum and other C_4 grasses, with narrower leaves expected to have lower g_s but higher iWUE [9, 21, 22]. LW and stomatal traits are extremely heritable traits in sorghum and can be screened more easily compared to using gas exchange equipment [14, 23, 24]. Our second aim was to investigate the degree of anatomical and morphological control on stomatal kinetics in sorghum.

Stomatal responses are also highly dependent on leaf water status, such as changes in leaf water potential (ψ_{leaf}) [25, 26]. Plants regulate ψ_{leaf} in response to soil water deficit following either an isohydric (conservative or sensitive) hydric pattern, where stomata close at high ψ_{leaf} to avoid excessive tension in xylem conduits and preserve ψ_{leaf} closer to soil water potential, or anisohydric strategy that disregards decreases in ψ_{leaf} in order to maintain g_s and CO_2 uptake [27, 28]. These two strategies can have implications for iWUE, as conservative plants can have higher iWUE due to their stomatal sensitivity to water stress (WS), at the expense of carbon accumulation which can be higher in less-sensitive

anisohydric plants [25]. This hydraulic trade-off is typical of plant resource-use economics, and likely can be reflected in dynamic traits such as stomatal kinetics as well as steady-state traits [25]. Our third aim was to expand our understanding of the physiological underpinnings of iWUE, including $iWUE_{dyn}$, to incorporate hydric behavior and its influence on stomata, and explore its diversity in a key crop [29, 30].

To link these physiological mechanisms to possible genetic regions for further exploration, we conduct our measurements on a population of sorghum from parental lines harboring different haplotypes associated with different aquaporin (AQP) alleles. We have identified differences in steady-state iWUE for these lines previously [14], and attempt now to see if the same lines emerge with differences in stomatal kinetics. AQPs or related genes have been shown to play an important role in regulating stomatal kinetic responses to changing environments and influencing *iWUE*, including in sorghum [31–34]. We analyzed differences in $iWUE_{dyn}$ and explored its relationship to stomatal and hydric behavior under WS conditions and saw whether anatomical variables played a role in determining those responses. Our main hypotheses were: 1) $iWUE_{SS}$ and $iWUE_{dyn}$ will correlate among the genotypes under well-watered (WW) and WS conditions; 2) this will be the result of faster closing of stomata during light decrease, but slower opening of stomata when light intensity increases; 3) high iWUE will correlate with increased isohydricity and stomatal sensitivity to reductions in leaf water potential 4) and characterized by lower SD and higher SS.

2. Materials and methods

Genotype selection, growth conditions, water stress treatment and AQP analysis were conducted as described in [14] but we provide a detailed summary below.

2.1. Genotype selection

The genotypes used here are part of a nested association mapping (NAM) population [35] (Method S1). Genotypes used in our study came from a sorghum NAM population that comprises an elite parental line R937945-2-2 (Recurrent Parent, RP) crossed with > 100 exotic lines with geographical or racial diversity (Non-Recurrent Parent, NRP). The F_1 progeny were backcrossed with the elite parent to produce BC_1F_1 populations. BC_1F_1 genotypes compromise 22%−25% exotic (NRP) line genome with the rest being RP background (Fig. S1). Individual BC_1F_1 populations are genotyped using high density single-nucleotide polymorphism (SNP) markers providing profiles of the exact exotic chromosomal segments, giving us information on what genes are coded for in the 22%−25% NRP portion of the genome, and what genes are coded for in the remaining RP section of the genome. This population was screened using 8 AQP genes to select lines carrying non-synonymous SNP alleles of those genes. Specifically, the subpopulation was screened to identify individual lines with chromosomal segments harbouring the elite (RP) AQP allele (RP-Haplotype) or the exotic (NRP) AQP allele (NRP-Haplotype) of a specific AQP. The final 89 lines chosen were derived from 5 exotics (NRPs) containing a mix of geographical origins (Table S1). This approach allowed us to create subpopulations within the 89 genotypes through focusing on one of the 8 AQPs, with each subpopulation containing two sets of genotypes, a set (> 5) of genotypes containing the RP-haplotype for that AQP, and a set containing the NRP-haplotype. Hence, any phenotypic difference when comparing RP or NRP haplotypes associated with a certain AQP may be due to the specific AQP allele that characterize the RP or NRP haplotype or from the accompanying genes from that chromosomal segment (haplotype).

2.2. Plant culture

Eight litre cylindrical pots were used to allow ample space for root development before implementation of the water stress treatment. The pots were adjusted to similar weight (1.5 kg) by adding gravel (100–300 mm diameter), then the same amount of soil was added to all pots. The potting mix was made of soil, sand and decomposed bark. It had large particle size for good drainage and root development. Granulated fertiliser (Osmocote Plus Organics All Purpose Fertiliser, Scotts Miracle-Gro Company, Marysville, Ohio) was pre-mixed with the soil, with more fertiliser added in the lower half of the pot as roots developed with plant growth. Seeds were directly sown into the upper soil layer. 2–3 seeds were planted per pot and when germinated plants were 7–10 d old, the healthiest looking plant was kept and the others uprooted, leaving one plant per pot. Plants germinated and grew in a naturally lit, controlled-environment greenhouse (Plexiglas Alltop SDP 16; Evonik Performance Materials, Darmstadt, Germany) at the Hawkesbury Institute for the Environment, Western Sydney University, Richmond, New South Wales, Australia (-33.612032, 150.749098).

The ambient temperature was set at 30 °C during the day period, with night temperatures set at 18 °C. There was a 2 h period at 24 °C between the temperature transitions. The day temperature started at 8 a.m., and night temperature at 8 PM, when sunrise was about 5 to 6 a.m. and sunset at 7–8 PM, reaching midday maximums of ~35 °C and midday relative humidity of 40%–50% (Figs. S2, S3). CO_2 concentration was kept at ambient levels. Due to the large number of plants, we needed three identical and adjacent greenhouse chambers (8 m long × 3 m wide × 5 m tall), which contained both well-watered and water stressed pots, and pots were swapped between the three chambers fortnightly during growth in a randomised fashion. Chamber conditions were monitored via a data logger (Tinytag plus 2, Omni Instruments, Dundee, UK) hung in the middle of the room at 2 m height. Light levels were monitored occasionally using a light meter and were 1500 µmol m^{-2} s^{-1} at midday on sunny days at plant height level at measurement time (~2 m from the ground).

2.3. Watering treatments

All plants were well-watered the first six weeks of growth when half of the plants by genotype were subjected to water stress (WS) and the other half continued under well-watered conditions. Field Capacity (FC) was determined using pot weighing (see Method S1). After 6 weeks of growth, watering was withheld from half of the pots (WS, water stress treatment), while the other half continued to be watered at FC (WW, well-watered treatment). Stomatal conductance was monitored in WS plants until it reached around 0.1 mol m^{-2} s^{-1} or less at saturating light, with the plant also showing signs of wilting. When conductance reached the required level, and signs of wilting appeared, the volumetric soil water content was about 5% for most pots. At this point, we measured pot weight as described in Method S1 to establish the amount of water lost by evapotranspiration in a day (about 50 mL). Three folds this amount of water, equivalent to total plant transpiration during the day in the WS treatment for three d, was added every three d to the WS pots. Hence, plants under WS got just enough water for replacement of water loss via daytime evapotranspiration, and we ensured that the water status of WS plants was not influenced by recent watering by delaying the measurements to the third day after watering. There were three replicates (pots) per genotype and water treatment. Hence, each genotype had 6 pots total, with 3 for each treatment ($n = 3$), except for the elite parent R937945-2-2 (the RP) which had 6 pots per treatment ($n = 6$). Some genotypes were extremely stressed during our WS treatment and were giving very small stomatal conductance values (~0.01 mol m^{-2} s^{-1}), and hence had to be discounted from the sampling, yielding only 61 genotypes for the WS

treatment compared to 89 for WW. More detailed information about the genotype selection, plant culture and watering can be found in the supplementary material.

2.4. Leaf gas exchange

Midday leaf gas exchange rates were measured between 10 a.m. and 2 p.m. on sunny days. The photoperiod was 14–15 h, and solar midday was around 1–1:30 p.m. A Li-6400XT infra-red gas analyser with a LED light source and an area of 6 cm^2 (LiCor Biosciences, Lincoln, Nebraska, USA) was used to obtain light-saturating rates of CO_2 assimilation (A_n), stomatal conductance to water vapour (g_s) and transpiration flux (E); cuvette conditions were set at: 30 °C block temperature, flow rate of 500 $\mu mol\ m^{-2}\ s^{-1}$, photosynthetic photon flux density (PPFD) of 2000 $\mu mol\ m^{-2}\ s^{-1}$ (10% blue light), ambient CO_2 concentration set to 400 $\mu mols\ m^{-2}\ s^{-1}$ using CO_2 cylinder mixer, and relative humidity of 40–60%. The leaf was inserted into the gas exchange cuvette under those conditions avoiding the midrib and with the entire 6 cm^2 area of the cuvette filled. The leaf was left to acclimate for ~10 min to those conditions until gas exchange and CO_2 concentration in the sub-stomatal cavity (intercellular CO_2, C_i) stabilised. Steady state intrinsic water use efficiency (iWUE$_{SS}$) was calculated as the ratio of A_n to g_s. All measurements were taken from the middle of the youngest fully expanded leaf (YFEL) of the plant, corresponding to the 9th–12th leaf depending on genotype. Ambient light level at the YFEL was around 1500 $\mu mol\ m^{-2}\ s^{-1}$.

The same YFEL leaf was subjected to a special light regime program that was created in the Li-6400XT. After stabilizing at 2000 $\mu mol\ m^{-2}\ s^{-1}$, the light regime started with (1) maintaining 2000 μmol photons $m^{-2}\ s^{-1}$ for 4 min, followed by (2) 2 min at 100 $\mu mol\ m^{-2}\ s^{-1}$, then (3) back to 2000 $\mu mol\ m^{-2}\ s^{-1}$ for 4 min. Measurements were logged every 5 seconds. The short time periods of light were chosen mainly because of the fast kinetics of sorghum, with most response time constants to stomatal opening in sorghum among the majority of screened genotypes being less than 5 min and usually as low as 1 min [11, 13, 36], and for stomatal closing is always less than 2 min and usually less than 1 min [11, 13]. Furthermore, this enabled us to screen the large number of plants we were growing, and these timings are also more representative of a sun "fleck" in the field under subtropical conditions. In addition, as McAusland et al. [13] explain, most of the inter-specific variation in respect of stomatal behavior is found in the initial part of the response (and hence why the lag parameter (see below) might play an important part). The model is still able to fit even if the steady state was not reached for some curves (as it was in [13]). We have used this protocol on sorghum genotypes before in a different experiment and it was sufficient [37]. To calculate stomatal kinetics parameters, the model developed in Vialet-Chabrand et al. [38] and refined in McAusland et al. [13] was used:

$$g_s (t) = (g_{ss} - g_0)e^{-e^{\frac{(\lambda - t)}{k}}} + g_0$$

(1)

Where g_{ss} is final steady g_s, g_0 is the starting g_s at the moment of light change and t is time. The time constant (k) for stomatal opening (k_{open}) and closing (k_{close}), an estimate of the time taken for stomata to get to 63% of the final value [38], was estimated by the model, as well as the lag term λ that describes the time lag in g_s response after the light change and before the response curve starts. The term is only presented for the opening phase as no lag was observed during transition from high-to-low light intensity and observed before especially in grasses and crops [11, 13]. The light cycles include distinct phases of high-to-low and low-to-high light intensity changes. The average iWUE and g_s during the

light phases were obtained separately for the low-light closing phases (iWUE$_{dyn-close}$, g_{sclose}) and the high-light opening phases (iWUE$_{dyn-open}$, g_{sopen}). Excess transpiration (ΔE) due to slow stomatal closing was calculated as the time integrated difference between the initial and final E during transition from high to low light intensity. Forgone photosynthesis (ΔC) due to slow stomatal opening was calculated as the difference between the integral of A_n under the observed curve and the integral if maximum A_n were reached instantaneously during transition from low to high light intensity [11].

Finally, classic light response curves were collected as well on the studied plants but were only utilized to calculate the slope of the A_n vs. g_s relationship, the so called "marginal water cost of carbon" (g_1), an estimate of iWUE that is based on the optimality principle of stomatal behavior [39]. The light curve was conducted at the same CO_2, humidity and temperature conditions as mentioned. The semi-empirical model illustrated by [40] was used to calculate g_1 as:

$$g_s = g_r + 1.6\frac{g_1 A_n}{\sqrt{VPD \times C_a}}$$

(2)

Where g_r is residual stomatal conductance, taken as 0.01 mol m^{-2} s^{-1}, VPD and C_a are vapour pressure deficit and ambient CO_2 in the gas exchange chamber, and A_n is carbon assimilation rate.

2.5. Leaf water potential and degree of anisohydry

A leaf adjacent to the gas exchange leaf was used to measure midday leaf water potential (Ψ_{leaf}) using a Scholander-type pressure chamber (Model 1505D Pressure Chambers, PMS Instrument Company, Albany, Oregon, USA). Pre-dawn leaf water potential ($\Psi_{pre-dawn}$) was sampled on different leaves before sunrise, usually taking leaves in the lower canopy. In each case, the leaf was cut at the ligule and placed in a plastic bag that was exhaled into before sealing. The bags were stored in ice boxes, then transported from the greenhouse to the lab where leaf water potentials were measured within 1–2 h of excision. The Degree of anisohydry (ξ), which quantifies the spectrum between isohydry to anisohydry as it increases, was measured based on [27] as $\Psi_{leaf} / \Psi_{pre-dawn}$.

2.6. Stomatal sensitivity to drops in Ψ_{leaf}

Despite calculating g_1 and ξ, we still wanted to link stomatal and hydraulic sensitivity together. We used theory and equations from [28] to estimate the sensitivity of g_s operation to change in Ψ_{leaf} using equation 3. We assumed a sigmoidal response of g_s from its theoretical maximum (considered here as g_{smax}) at $\Psi_{pre-dawn}$ to its operational midday g_s at Ψ_{leaf}.

$$g_s = \frac{g_{smax}}{1 + e^{\beta(\psi_{pre-dawn} - \psi_{leaf})}}$$

(3)

The term (β) represents the sensitivity of stomata to reductions in Ψ_{leaf}, with high β meaning increased sensitivity of g_s (isohydric) and low β is decreased sensitivity (anisohydric). Because we used g_{smax}, we calculated β only genotypes with anatomical data were assessed.

2.7. Leaf morphology

Leaf width (LW) was measured at the same leaf area where gas exchange measurements were made. After measuring gas exchange and on the same portion of the leaf, we collected three leaf discs of 0.5 cm^2 each to measure leaf mass per area (LMA). Leaf discs were placed inside an oven at 65 °C for 48 h to measure dry weight. LMA was calculated as dry weight / leaf discs area (g m^{-2}). Plants were harvested after 95–100 d, and total aboveground biomass was measured after drying in an oven at 40 °C for 10 d before measuring dry biomass.

2.8. Stomatal anatomy

Two negative impressions using nail varnish were taken of the middle portion of the same gas exchange leaf, one impression in each side of the midrib, and for both adaxial and abaxial sides of the leaf. The impressions were attached to a microscope slide using transparent tape and imaged under a light microscope (Axio Scope.A1, Carl Zeiss Microscopy GmbH, Jena, Germany) at × 10 magnification. Photomicrographs were analysed using Image J [41]. Three areas between the 2nd and 3rd major longitudinal veins of 0.6 mm^2 each were selected to count the stomata. Stomatal density (SD) was calculated as the number of stomata per unit area by adding SD of adaxial and abaxial side. This is the SD discussed from here on. Within each area where SD was calculated on each side, ten stomata were randomly selected to measure the following variables: Stomatal size (SS) was calculated by multiplying stomatal width (W_s, including two guard cells and two subsidiary cells) by guard cell length (L_s) and expressed in μm^2 as shown in [9, 22]. Maximum pore aperture (A_{max}) and maximum theoretical anatomical stomatal conductance (g_{smax}) were calculated as described in Franks and Farquhar [42] and Al-Salman et al. [11]. Shown *SS* and A_{max} were the average of the two sides of the leaf. A lower number of genotypes (45) was sampled here due to time and physical constraints, but all haplotypes were still represented robustly (Tables S4 and S5).

2.9. Genetic variation

Broad-sense heritability was calculated as in [43]:

$$H_b = \frac{\sigma_g^2}{\sigma_p^2} \tag{4}$$

where σ_g^2 and σ_p^2 are the genotypic and phenotypic variances respectively. σ_g^2 was obtained as the sq. of mean from the ANOVA output. σ_p^2 was calculated as:

$$\sigma_p^2 = \sigma_g^2 + \frac{\sigma_{g \times treatment}^2}{\text{number of treatments}} + \frac{\sigma_e^2}{\text{number of replicates}}$$

(5)

where $\sigma_{g \times treatment}^2$ and σ_e^2 are the genotype × treatment interaction and error variances respectively. $\sigma_{g \times treatment}^2$ was obtained as the mean squared of the genotype × treatment interaction and σ_e^2 was obtained as the sq. of mean residual error. Because the heritability analysis encompasses both treatments, the number of replicates was standardized as 5 (as opposed to 6; 3 WW and 3 WS) to account for genotypes not in both treatments.

2.10. Statistical analyses

Statistical analysis and data visualization were performed using R software (R Core Team (2020) https://www.R-project.org/). Normality was checked by plotting a generalized linear model and inspecting residual plots. Analysis of variance (ANOVA) and multiple ANOVA (MANOVA) was carried out using linear mixed-effects models

(package nlme), with replicate random variable for genotype comparisons and with replicate and genotype as the random factor for Haplotype comparison, with AQP Haplotype × Water Treatment being the fixed variables to get the P-value associated with the model. Variance within groups was performed afterwards using a *posthoc* Tukey test. Regression analysis was carried out in R using linear modelling (lm function). A Pearson product moment correlation analysis was performed to test statistical significance of relationships at $P < 0.05$ and obtain correlation coefficient R. If both WS and WW treatments show the same directional correlation, then we show the global correlation only.

3. Results

3.1. Large variation in dynamic *iWUE* and stomatal responses to light in sorghum genotypes

We found a large variation of stomatal kinetic parameters among the 89 genotypes measured. $iWUE_{dyn-close}$ had a fold change within genotypes of 4.16 under WW and 5.31 under WS, while $iWUE_{dyn-open}$ had a fold change of 1.62 and 1.53 under WW and WS respectively (Table 1). Both increased significantly under WS (Table 1). k_{open} but not k_{close} experienced a significant overall ($P > 0.0001$) increase under WS, meaning that stomata opened slower under WS (Table 1). Both time constants experienced large variations under both WW and WS. Broad sense heritability (H_b) of k_{close} and k_{open} was 0.58 and 0.56 respectively, but $iWUE_{dyn-close}$ and $iWUE_{dyn-open}$ had higher H_b of ~0.7 (Table 1). SD and SS experienced no significant changes between treatments, but there was variation between the genotypes with fold changes from around 1.7 at WS and up to 4 for SD at WW, with both parameters showing H_b of 0.72 (Table 1). Means and standard errors of all the parameters sampled in this paper are in Tables S4 and S5

Table 1 – Means (± Standard error) and *P*-values from a mixed effect ANOVA comparing treatments and Broad-sense heritability (H_b) of the main (WW, well-watered and WS, water stress).

Parameter	WW	WS	P-value	Fold change WW (%)	Fold change WS (%)	H_b
SD	196.68 (7.67)	185.26 (4.81)	0.307	4.15	1.79	0.72
SS	529.77 (13.22)	550.25 (13.18)	0.262	2.05	1.69	0.72
k_{close}	1.43 (0.21)	1.08 (0.1)	0.215	61.4	42.4	0.58
k_{open}	1.1 (0.05)	1.76 (0.14)	< 0.0001	11.6	14.3	0.56
$iWUE_{dyn-close}$	80.88 (2.39)	141.99 (5.97)	< 0.0001	4.16	5.31	0.72
$WUE_{dyn-open}$	156.7 (1.61)	179.66 (2.31)	< 0.0001	1.62	1.53	0.69

SD, stomatal density (mm^{-1}); SS, stomatal size (μm^2); k_{close}, time-constant of stomatal closure (min); k_{open}, time-constant of stomatal opening (min); $iWUE_{dyn-close}$, water use efficiency averaged over stomatal closure ($\mu mol\ CO_2\ mol^{-1}\ H_2O$); $WUE_{dyn-open}$, water use efficiency averaged over stomatal opening ($\mu mol\ CO_2\ mol^{-1}\ H_2O$);

3.2. Dynamic and steady-state *iWUE* correlate to each other and are influenced by slower stomatal opening but not quicker closing

We sought to confirm that higher $iWUE_{ss}$ is related to higher iWUE under dynamic light conditions, which was the case for both low-to-high and high-to-low light intensity transitions (Fig. 1A, B). Higher $iWUE_{ss}$ and $iWUE_{dyn}$ came with significant increase in k_{open} (Fig. 2A; Table S2), but not with reduction in k_{close} (Table S2), partially rejecting hypothesis 2. Higher $iWUE_{ss}$ and k_{open} were expected to lead to greater potential loss of carbon assimilation (ΔC) but instead correlated negatively with ΔC (Fig. 2B; Table S2). Nevertheless, small k_{open} came with significantly increased stomatal lag time (λ) (Fig. 2C). This lag slows the photosynthetic response, and combined with lower mean A_n of the slower-stomata genotypes (Table S2), led to decreasing ΔC with shorter λ (Fig. 2D).
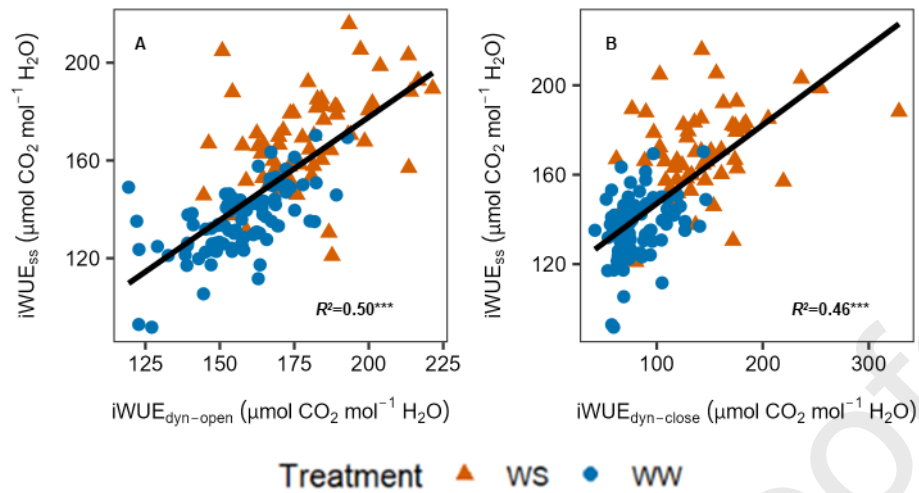
Fig. 1 – Relationship between steady state and dynamic water use efficiency (*iWUE*) in sorghum. Gas exchange measurements were taken during midday on the youngest fully expanded leaf. Steady state values were taken at 2000 µmols m^{-2} s^{-1} of light and 400 µmols m^{-2} s^{-1} CO_2 (see Materials & Methods). The transition from low-to-high light was conducted from 100 to 2000 µmols m^{-2} s^{-1}, with the reverse for the high-to-low light. Dynamic iWUE was the ratio of average carbon assimilation to average stomatal conductance over the period of light transition. Each dot in the scatter is the mean for one genotype for that treatment (n = 3). Correlation coefficients (R^2) are the result of a Pearson correlation test, ***, P < 0.001. Blue dots are for well-watered, red for water-stressed. (A) Steady-state iWUE ($iWUE_{ss}$) vs. Dynamic iWUE during low-to-high light transition ($iWUE_{dyn-open}$); (B) $iWUE_{ss}$ vs. Dynamic iWUE during high-to-low light transition ($iWUE_{dyn-close}$). Standard error can be found in Tables S4 and S5.
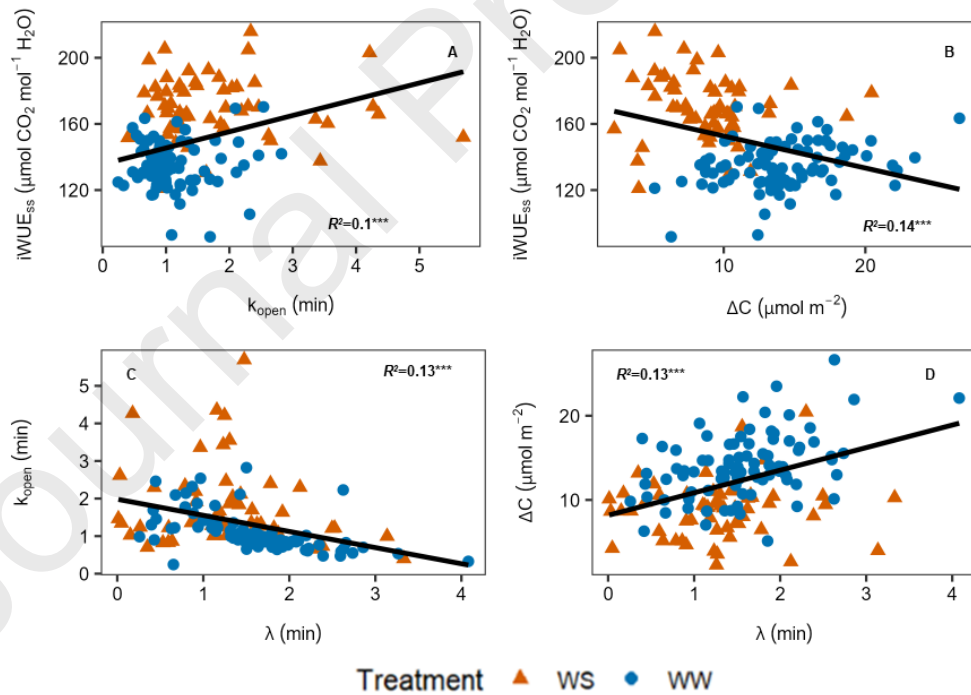

Fig. 2 – Relationship between steady state water use efficiency and stomatal kinetic parameters in sorghum. Gas exchange measurements were taken during midday on the youngest fully expanded leaf. Steady state values were taken at 2000 µmols m^{-2} s^{-1} of light and 400 µmols m^{-2} s^{-1} CO_2 (see Materials & methods). The transition from low-to-high light was conducted from 100 to 2000 µmols m^{-2} s^{-1}, with the reverse for the high-to-low light. Calculation of stomatal kinetic parameters was done using modelling as described in Materials & methods. Each dot in the scatter is the mean for one genotype for that treatment ($n = 3$). Correlation coefficients (R^2) are the result of a Pearson correlation test, ***, $P < 0.001$. Blue dots are for well-watered, red for water-stressed. (A) Steady-state iWUE ($iWUE_{ss}$) vs time-constant of stomatal opening (k_{open}); (B) $iWUE_{ss}$ vs Forgone photosynthesis (ΔC); (C) k_{open} vs time-lag constant of stomatal response (λ); (D) ΔC vs. λ. Standard error can be found in Tables S4 and S5. (NB, faster stomata is defined as low k_{open}).

3.3. Stomatal anatomy plays a minor role in sorghum gas exchange kinetics, but leaf width can be an indicator of leaf carbon-water balance

Stomatal anatomical traits did not have an impact on stomatal kinetic speed but were weakly correlated with steady state and dynamic g_s (mean g_s during light transition; i.e. overall capacity) (Table S2), partially rejecting hypothesis 4. Maximum anatomical conductance to water vapor (g_{smax}) did correlate weakly but significantly with increased ΔC and ΔE (Fig. 3E, F), highlighting a possible integrated anatomical influence on iWUE. Among the morphological traits assessed, we instead found a strong influence of leaf width (LW) on stomatal anatomy and leaf gas exchange, and a weaker one for stomatal kinetics. LW correlated positively with g_{smax} and SS (Fig. 3A, B), as well as steady state A_n and g_s (Table S2), and ΔC (Fig. 3D). LW also correlated negatively (significantly but weakly) with k_{open} (Fig. 3C). Leaf length (LL) correlated with LW positively, as well as SS, hinting at a general increase in leaf area along with stomatal size (Table S2).
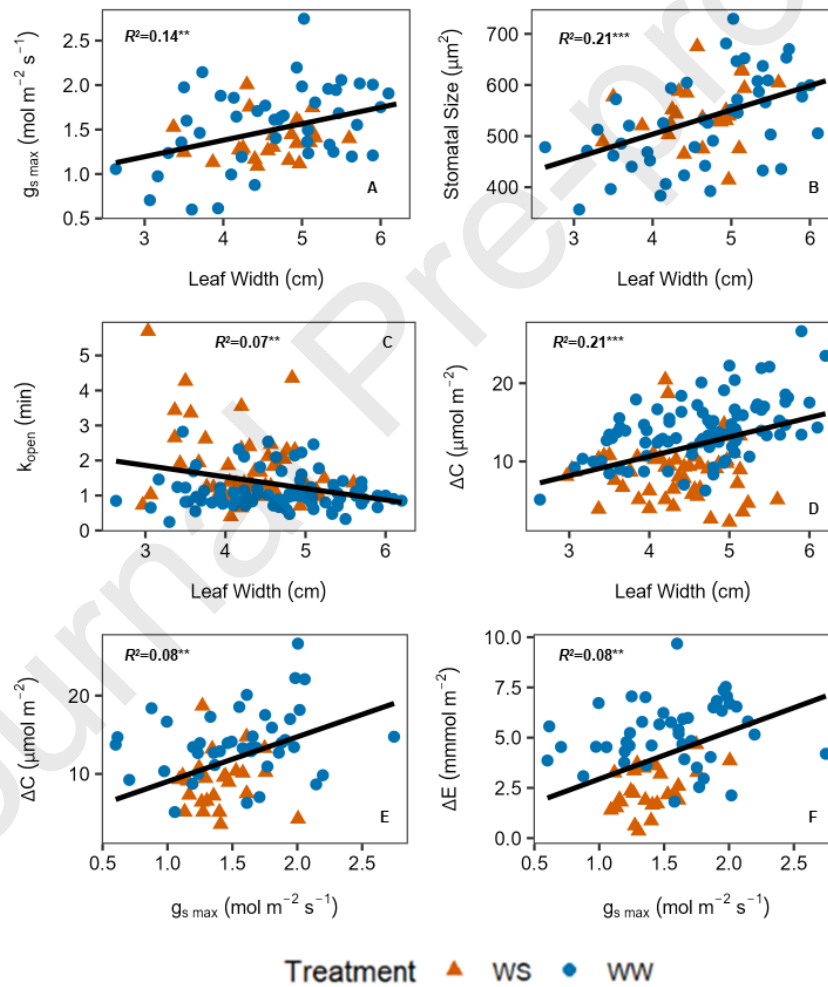

Fig. 3 – Relationship between stomatal kinetics and stomatal and leaf morphology in sorghum. Gas exchange measurements were taken during midday on the youngest fully expanded leaf, at 400 µmols m^{-2} s^{-1} CO_2 (see Materials & methods). Transition from low-to-high light was conducted from 100 to 2000 µmols m^{-2} s^{-1}, with the reverse for the high-to-low light. Calculation of stomatal kinetic parameters was done using modelling as described in Materials & methods. A strip spanning the width of the leaf at leaf length divided by 2 was used to measure anatomical and morphological characteristics. Each dot in the scatter is the mean for one genotype for that treatment ($n = 3$). Correlation coefficients (R^2) are the result of a Pearson correlation test, **, $P < 0.01$; ***, $P < 0.001$. (A) Anatomical maximum stomatal conductance (g_{smax}) vs leaf width; (B) Stomatal size vs. leaf width; (C) Time-constant of stomatal opening (k_{open}) vs leaf width; (D) Forgone photosynthesis (ΔC) vs leaf width; (E) ΔC vs. g_{smax}; (F) Excess transpiration (ΔE) vs. g_{smax}. Standard error can be found in Tables S4 and S5.

3.4. Stomatal kinetics can be linked to hydric behavior, and is underpinned by stomatal sensitivity to leaf water potential

Stomatal kinetics are influenced by leaf water status, so we measured the degree of anisohydry (ξ), a parameter that describes the tendency of plants to regulate g_s to maintain leaf water potential (Ψ_{leaf}) closer to soil water potential (see Materials & methods). Large ξ is indicative of a spender or non-conservative use of water, and hence a high ξ was expected to correlate with lower iWUE (Table S2), but also with faster stomatal opening during low to high light intensity transition (lower k_{open}). While we did find the latter relationship, it was very weak unless considering the water stressed plants (R^2=0.06, Fig. 4A). ξ correlated positively with ΔC and ΔE (Fig. 4B, D), as well as g_1, g_{smax} and g_s (Fig. 4C, E, F).
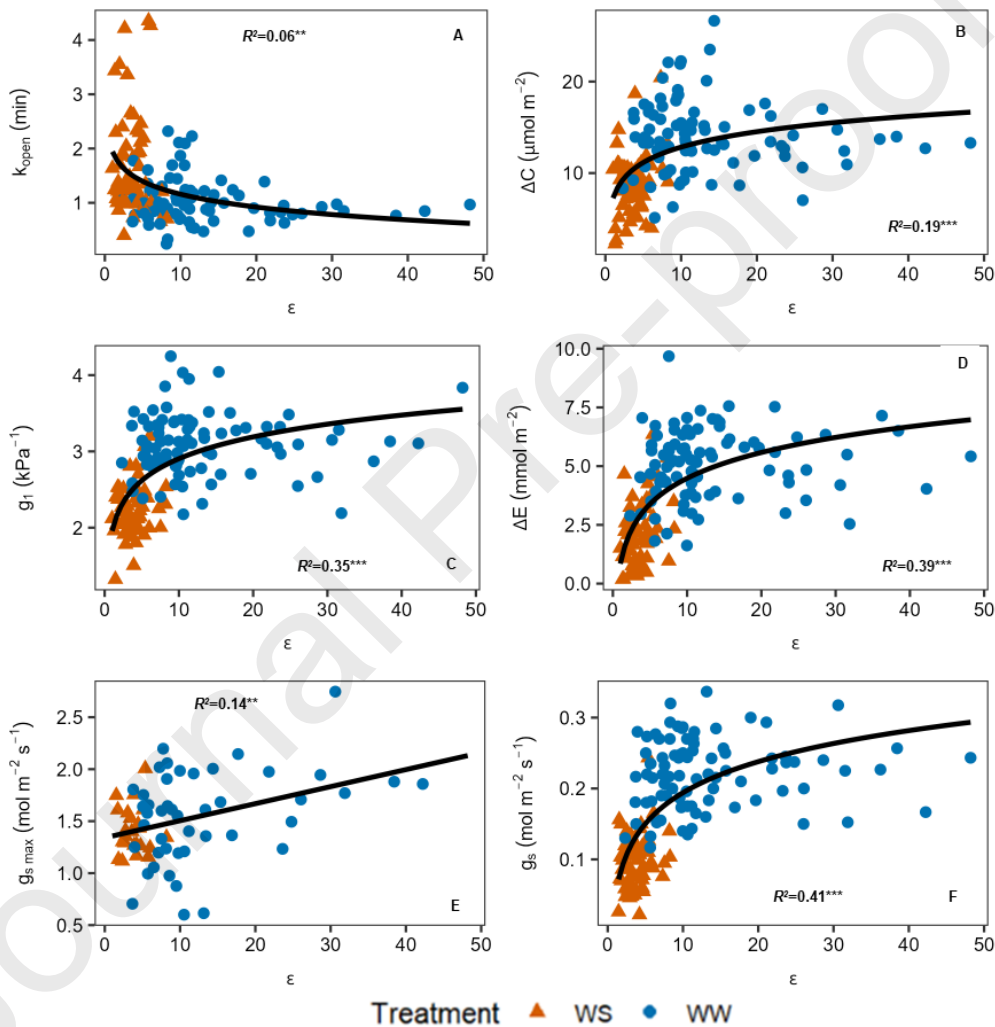

Fig. 4 – Relationship between hydric behavior and stomatal behavior in sorghum. Gas exchange measurements were taken during midday on the youngest fully expanded leaf. Steady state values were taken at 2000 μmols m^{-2} s^{-1} of light and 400 μmols m^{-2} s^{-1} CO_2 (see Materials & methods). Transition from low-to-high light was conducted from 100 to 2000 μmols m^{-2} s^{-1}, with the reverse for the high-to-low light. Calculation of stomatal kinetic parameters was done using modelling as described in Materials & methods. Degree of anisohydry was calculated as the ratio of midday to pre-dawn leaf water potentials. For pre-dawn water potential, leaves from lower down the canopy were sampled into a plastic bag in a coolbox and taken to the lab. For midday water potential, the leaf adjacent to that used for gas exchange was sampled into a wet paper bag and into a coolbox, transported to the lab and measured within 2 h. Midday leaves were sampled during the same diurnal gas exchange period on sunny days. Each dot in the scatter is the mean for one genotype for that treatment (n = 3). Correlation coefficients (R^2) are the result of a Pearson correlation test, **, $P < 0.01$; ***, $P < 0.0001$, or from the exponential fit of the model. (A) Time-constant of stomatal opening (k_{open}) *vs* degree of anisohydry; (B) Forgone photosynthesis (ΔC) *vs* degree of anisohydry; (C) Slope coefficient of stomatal response to photosynthesis and VPD (g_1) vs. degree of anisohydry; (D) Excess transpiration (ΔE) *vs* degree of anisohydry; (E) Anatomical maximum stomatal conductance ($g_{s\,max}$) *vs* degree of anisohydry; (F) Stomatal conductance (g_s) vs. degree of anisohydry. Standard error can be found in Tables S4 and S5.

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In summary, we found that more isohydric plants (lower ξ, closer to 1) slowed stomatal opening although with little penalty in terms of carbon gain due to the low achieved A_n at high light intensity, but lost less water (low ΔE), and displayed more optimal stomatal behaviour (g_1) under water stress. Then, we wanted to confirm the link between anisohydry with reduced stomatal sensitivity to drought by calculating g_s sensitivity factor to drops in water potential (β) (Fig. 5E). β was indeed significantly and positively correlated with iWUE$_{SS}$ and negatively with ξ and g_1 (Fig. 5A, C, D respectively).
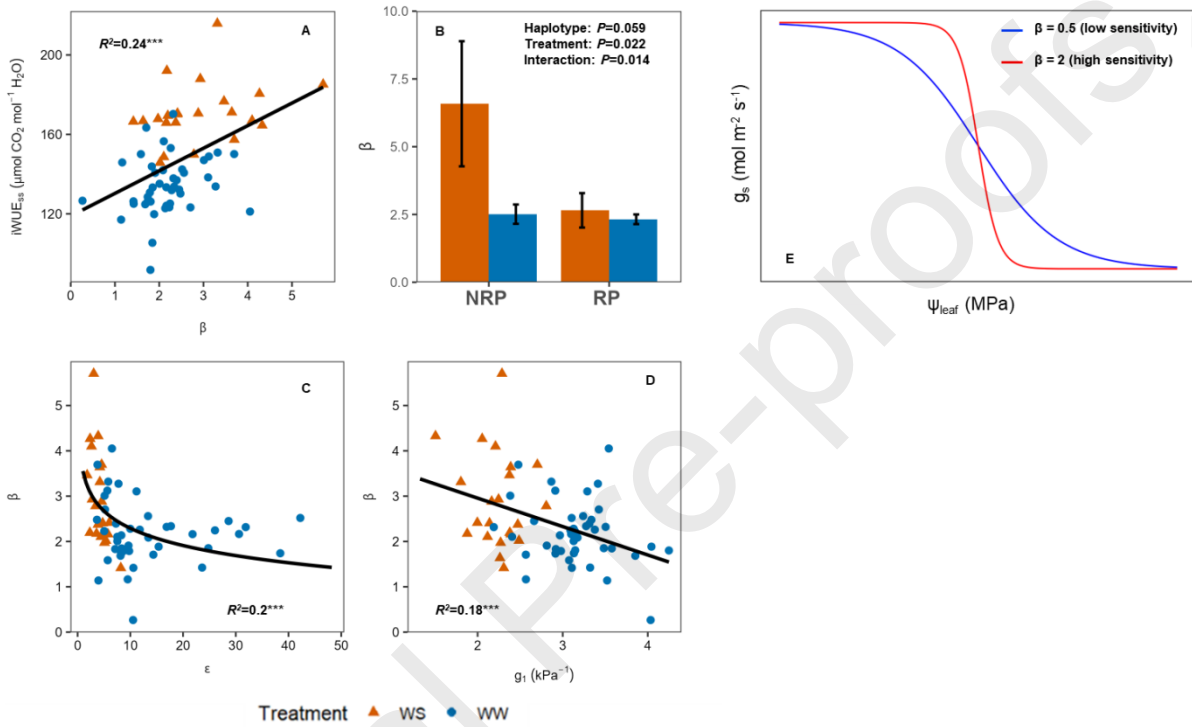

Fig. 5 – Variation in stomatal conductance sensitivity to water potential (β) between SbTIP3.2 haplotypes and its relationship to hydric behavior and stomatal optimality. For (B), the bar represents the mean of all individual replicates belonging to the genotypes of that population (n = 5–21). Statistics shown are the result of an ANOVA test and post-hoc Tukey test. Each population (RP & NRP) refers to a set of genotypes that either inherited the AQP haplotype block from the elite parent (RP) or from the exotic parent (NRP). Blue is well-watered, red is water-stressed. For (A), (C–D), Gas exchange measurements were taken during midday on the youngest fully expanded leaf. Steady state gas exchange values were taken at 2000 μmols m^{-2} s^{-1} of light and 400 μmols m^{-2} s^{-1} CO_2 (see Materials & methods). Degree of anisohydry (ξ) was calculated as the ratio of midday to pre-dawn leaf water potentials. For pre-dawn water potential, leaves from lower down the canopy were sampled into a plastic bag in a coolbox and taken to the lab. For midday water potential, the leaf adjacent to that used for gas exchange was sampled into a wet paper bag and into a coolbox, transported to the lab and measured within 2 h. Midday leaves were sampled during the same diurnal gas exchange period on sunny days. Each dot in the scatter is the mean for one genotype for that treatment (n = 3). Correlation coefficients (R^2) are the result of a Pearson correlation test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ or from the exponential fit of the model. Blue bars are for well-watered, orange for water-stressed. (A) Steady-state intrinsic water use efficiency (iWUE$_{SS}$) vs. β; (B) Variation in β in SbTIP3.2 haplotypes; (C) β vs. ξ; (D) vs slope coefficient of stomatal response to photosynthesis and VPD (g_1) vs. β. (E) Demonstration of the response of stomatal conductance (g_s) to drops in water potential (ψ). The two colors represent two leaves with different sensitivities (β) to drops in ψ as the plant transpires with increasing light and temperature from dawn to midday.

3.5. Haplotypes associated with SbTIP3.2 influences key traits determining dynamic *iWUE*

The physiological strategies described above were investigated to find possible genetic links associated with certain inherited chromosomal regions (haplotypes), selected based on variation in 8 AQP alleles. Similar to our earlier study on determinants of iWUE ([14], we found only a significant effect for the haplotype carrying the SbAQP TIP3.2 between the elite (RP) and exotic (NRP) haplotype populations. The genotypes with the RP haplotype displayed more water spender characteristics (and lower iWUE), including lower k_{open} and higher λ (Fig. 6A, B),

higher ΔC and ΔE (Fig. 6C, D), as well as higher g_{smax} and SD (Fig. 6E, F) (see also Table S3). This does not suggest that the specific AQP is driver behind these differences, but that genes associated with it might be playing a role.
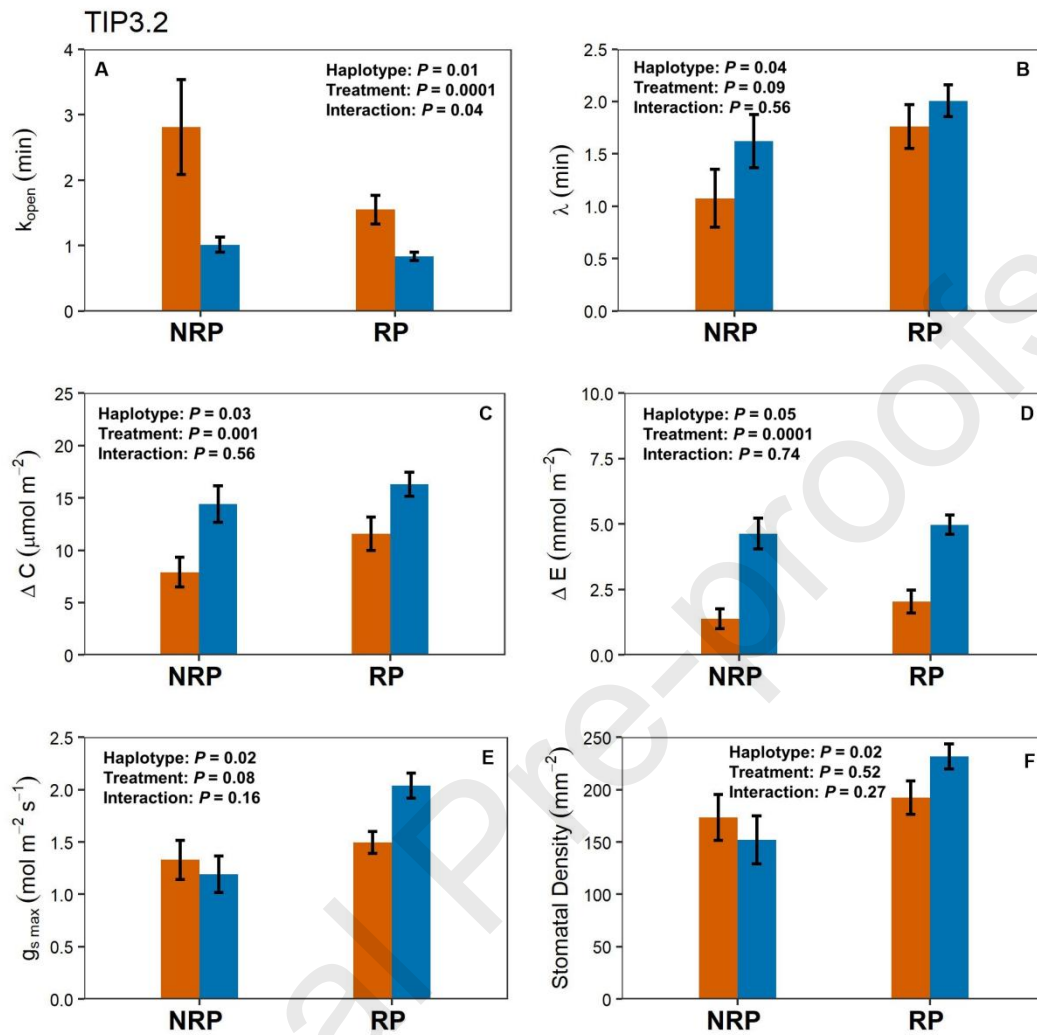

Fig. 6 – Bar charts showing the effects of SbTIP3.2 Aquaporin haplotype on key traits. The full analysis for all AQPs shown in Table S3. Each bar represents the mean of all individual replicates belonging to the genotypes of that population (n = 5–21). Statistics shown are the result of an ANOVA test and post-hoc Tukey test. Each population (RP & NRP) refers to a set of genotypes that either inherited the AQP haplotype block from the elite parent (RP) or from the exotic parent (NRP). Blue is well-watered, orange is water-stressed. (A) Time-constant of stomatal opening (k_{open}); (B) Time-lag constant of stomatal response (λ); (C) Forgone photosynthesis (ΔC); (D) Excess transpiration (ΔE); (E) Anatomical maximum stomatal conductance (g_{smax}) ; (F) Stomatal density (SD).

4. Discussion

Genotypes with leaves that respond faster to dynamic light environments are expected to increase crop WUE and potentially yield under water scarcity conditions. We screened for variation in stomatal kinetics to transient light intensity conditions in a subset of a sorghum NAM population with different exotic haplotypes and under WW and WS conditions. We found that 1) slower opening of stomata is linked to higher steady-state and dynamic iWUE and lower lag time before opening (λ) 2) stomatal kinetic time-constants were not associated with stomatal traits, but faster stomatal opening was associated with increased leaf width (LW) and is driven by anisohydric behavior and reduced drought sensitivity and 3) the haplotype carrying the AQP SbTIP3.2 might play a role in regulating stomatal kinetics, steady-state and dynamic iWUE, and hydric behavior.

4.1. Balancing CO_2 and H_2O exchange with drought sensitivity

The fast opening of stomata to maximize photosynthesis requires corresponding water loss through the pore. The tendency to favour carbon accumulation over water conservation has been associated with fast-growing water spending plants [44]. These plants usually function at high ψ_{soil} to ψ_{leaf} differentials in order to create pressure potentials that drive higher hydraulic conductance (K_{leaf}) in order to keep the stomata open and responsive [25,27]. This is shown by the large ξ of genotypes that experience higher g_s, lower k_{open} (Fig. 4) and higher $A_{n\text{-}open}$ (Table S2), and by the lower hydraulic resistance ($1/K_{leaf}$) of the same fast genotypes in our earlier publication [14]. The association of anisohydry with faster stomatal kinetics has been shown before in woody species [25], leading to reduced stomatal limitation of photosynthesis. But despite the apparent reduction in limitation, ΔC and k_{open} correlated negatively (Fig. 2). A likely reason is the influence of longer stomatal lag (λ) in the faster genotypes (Fig. 2D), which can increase stomatal limitation in the early part of the response. At the same time, those genotypes can have low non-stomatal limitations during the induction of C_4 photosynthesis [45], leading to significantly higher increase in A_n during light transitions while the stomata lags. The increased energetic cost and metabolic pools that power C_4 photosynthesis need time to build up [46, 47]. This high energy cost is likely reflected in the function of the dumbbell-shaped guard cells of sorghum, which are known as being more efficient and faster than their kidney-shaped counterparts. However, they require significant starch buildup (through photosynthesis) and breakdown [48] and the buildup of ions, which can create this lag that has been observed before [49]. Another possible reason is that the reduced sensitivity to leaf water status, which we estimate here via β, can contribute to delayed responses of fast genotypes, creating a trade-off. Overall. This finding provides intriguing possibilities about the role of internal leaf signals in activating stomatal responses [48, 50]. Also, while the genotypes contain haplotypes with many different genes and we only used the AQP alleles to differentiate them, a role for SbTIP3.2 here is still possible, as ion transfer in the guard cells can also be driven by activation of aquaporins and other ion channels [31, 32], and TIP3.2 is known as an H_2O_2 transporter, which is crucial for stomatal function [51, 52]. However, it is likely that other genes on the chromosomal region also played a role beyond the AQP genes. To confirm these findings, a part of future work is to determine gene expression and transcription levels for these haplotypes.

Achieving high g_s with fast stomata would require increased insensitivity to changes in leaf water status (Fig. 6). This means that leaves must develop hydraulic adaptations, and likely hints at a more robust hydraulic vasculature for these faster genotypes [53]. This can be characterized by higher vein densities, which are usually concurrent with higher SD [54]. Higher SD (and g_{smax}) correlated weakly with higher ΔC, and higher ΔC with faster stomata (Table S2), meaning that stomatal anatomy exerted this minor influence on stomatal speed likely through indirect effects such as vein structure. Recent studies found evidence that increasing vein density correlates with narrower leaves [9,55], but narrow leaves in our study were weakly associated with slower stomata (Fig. 3C). However, the same studies found that wider leaves accumulate a higher number of longitudinal veins with thicker vascular bundle and bundle sheath size, likely conferring vascular robustness and protection from embolism [56–58]. Indeed, in our study, we found that higher ΔC and lower k_{open} correlate with increased leaf thickness (Table S2), possibly indicating that to accommodate the faster kinetics and lower water potentials, plants develop leaves with denser and thicker mesophyll to increase the ratio of cross-sectional xylem surface area to leaf area via increasing thickness [25], significantly influencing the pattern of carbon investment in the leaf. Furthermore, these changes in the mesophyll can influence path lengths of water transport and reduce signalling pathways for the stomata [59]. This highlights the importance of integrating ecological concepts such as the economic spectrum [60] into crop improvement, which

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is already being picked up [61].

Consequently, these findings point at a possible reduced importance for anatomy and a larger role for integrated physiological processes that impact guard cell electrophysiology and photosynthesis. Several studies have concluded that reducing SS would increase the rate of solute and water transfer into the guard cell, causing faster stomatal response [16, 17, 62]. However, more and more evidence is accumulating that this relationship between "size and speed" is not strict, especially in crops [11, 19, 63]. In the case of C_4 graminoids like sorghum, SS is already quite large (~500 μm^2 in our study and up to 1000+ μm^2 in previous studies [9, 18]). Also, the subsidiary cells surrounding the guard cells and the dumbbell-shape can reduce the influence of size on the rate of solute transfer as the longitudinal shape of dumbbell stomata allows for large surface-area to volume contact with neighboring subsidiary cells, and the increase in guard cell length might even enhance this contact [64]. Hence, beyond anatomy and kinetics, genetic variation in stomatal physiology can play an important role in determining plant iWUE, as shown recently in rice [65]. These physiological requirements would also be different between a C_3 crop like rice and a C_4 crop like sorghum, with C_4 leaves consistently showing faster stomatal opening and closing speeds, likely enabled by specific energetic processes in guard cells and subsidiary cells which is underpinned by the various differences in the energetic requirements of C_4 compared to C_3 photosynthesis that we discussed above.

4.2. Implications for breeding for higher iWUE and crop improvement

Deconstructing physiological traits such as iWUE helps to identify novel genetically-underpinned traits to use for breeding and to uncover trade-offs that occur when breeding for desirable traits, something especially evident when considering iWUE [66]. For example, our observation that different genotypes display different hydric strategies expands the scope of breeding for iWUE into yet unexplored traits that underpin other physiological processes. Breeding for iWUE has encountered many bottlenecks and trade-offs, especially in C_4 crops like sorghum [15]. Understanding upstream processes such as stomatal behaviour and drought sensitivity lays the groundwork for understanding what trade-offs underpin iWUE under different $G \times E$ interaction. For example, the water-spending genotypes here might be suited to water-available environments with cloud cover, such as temperate latitudes, where their fast stomata help them achieve quicker transitions under high light and achieve higher photosynthesis and productivity. On the other hand, the water-conserving genotypes might be more suitable for sub-tropical or sunny environments with minimal sun flecks and less available water, offsetting lower photosynthesis rates by maintaining constant rates due to minimized environmental changes. This can lead to these genotypes requiring less irrigation because their low transpiration rates mean more water is conserved in the soil for longer, allowing the plant to keep growing and experience lesser fluctuations in hydric status (lower g_s correlated with decreased ξ and tighter regulation of Ψ_{leaf} by stomata (Fig. 4F)). Actually, narrow leaf sorghum genotypes displayed an instantaneous increase in WUE but also higher WUE over the plant's life cycle [67]. This conservative and high iWUE mechanism has been found in other sorghum varieties [68], where reduced transpiration improves relative water content and Ψ_{leaf}, maintaining growth rate and possibly offsetting any reductions in instantaneous photosynthetic rates brought upon by reduced g_s. The interaction of the environment with the stomata will be even more variable in the field, with factors such as wind (affecting boundary layer), light spectra (affecting activation of photosystems) and light quality (diffuse $vs.$ direct) as well as VPD all interacting to influence the degree of stomatal opening or closing [10,13]. Linking responses of stomata, and subsequently photosynthesis, to these environmental changes at different temporal scales to variation in yield under field conditions can provide clarity about the role leaf physiological

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processes play in determining crop productivity [15]. The environmental conditions in our glasshouse stimulated high evaporative demand (Figs. S2, S3), and hence our results have the potential to show what traits and processes are important in terms of drought resilience for sorghum.

Following on from that, we are not aware of a screen for stomatal kinetics that involves this number of genotypes and a water stress treatment for a single crop species. There have been attempts to incorporate these traits into large scale efforts using proxies such as thermal imagery in sorghum [69], or others using lower number of genotypes in order to establish relationships [11, 18, 19, 63], with the highest being 43 genotypes [36]. Hence, finding variation between closely related genotypes is promising, despite the non-exhaustive replication ($n = 3$). In terms of anatomy, the lack of impact of WS on SD and SS means that reductions in g_s due to growth under WS are due to changes in the active pore size of the stomata. Most studies on crops compare mutants with low SD as a way to conserve water and increase iWUE [70, 71]. However, there are fewer studies on how stomatal anatomy is different in genotypes under different watering conditions. Generally, those studies found that grasses increase SD and reduce SS as a common response to WS [72–74], while in dicots the responses were SD decreasing with WS [75,76], but others showing SD increasing [77]. In short, there is uncertainty, and it seems that physiological and biochemical factors affecting stomatal responses are probably more consistent than anatomy [74]. Finally, finding a correlation between LW (and leaf length) and SS (and g_{smax}) similar to earlier findings in sorghum [9] highlights that this coordinated development between leaf expansion and anatomy is probably genetically conserved and can be a focused selection goal to combine stomatal traits with traits (LW) that can affect canopy wide gas exchange and energy balance [21, 22], with direct ramifications for crop water and radiation use efficiency. We also acknowledge that these hypotheses need to be tested under variable field conditions to confirm their robustness and links to yield. The closest estimate to yield in our study is our measurement of panicle weight at maturity, and we found that it correlated positively with LW, LL, A_n, g_s, as well as ΔE (and hence maybe indirectly with faster stomata, Table S2). These correlations will need to be validated with better measures of yield but they do point towards the expected association of high water use and higher yield, which is a relationship that is proving hard to break [15]. We believe that incorporating extra eco-physiological indices such as the ones we explore in this paper will provide some pointers as to why this trade-off is important and enable us to plan future trials more robustly.

5. Conclusions

We followed our earlier screen [14] of iWUE in sorghum genotypes with different haplotypes by exploring the underpinnings of iWUE under fluctuating light. The results showed that stomatal anatomy exerts minimal control on stomatal kinetics and iWUE, but might have an indirect effect through its relationship with other anatomical factors as evidenced by the correlation with ΔC & ΔE. Stomatal kinetic behavior was underpinned by a trade-off between stomatal sensitivity to drought and water conservation versus reduced drought sensitivity that promotes water spending have higher photosynthesis rates. We found there is a possible link for these mechanisms with the chromosomal region harboring alleles of the SbTIP3.2 AQP. While we cannot confirm the specific contribution of this AQP yet, this opens the door for newer genetic determinants of iWUE that do not compromise productivity.

Data availability

The data generated and analysed for this study are available from the corresponding authors by request.

Declaration of competing interest

Appendix A. Supplementary data

Supplementary data for this article can be found online at http://doi.org/10.1016/j.cj.2020.xx.xxx.

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