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Bigger is not always better: Reducing leaf area helps stay-green sorghum use soil water more slowly

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ABSTRACT

Stay-green, a trait that confers delayed leaf senescence and improved grain yield under post-anthesis drought, has been associated with smaller canopies at flowering and increased water uptake during the post-flowering period. It has been shown that the main stay-green quantitative trait loci reduce leaf area via reduced tiller number and smaller leaves. To show that these canopy characteristics are directly linked to water savings, we grew near-isogenic lines with and without stay-green introgressions in large lysimeter pots and measured their weekly pre-anthesis water use and main-stem and tiller leaf area. Paradoxically, age-related senescence of lower leaves in stay-green lines was accelerated before flowering, contributing to their smaller leaf area at flowering. This process of reducing leaf area by shedding old leaves lower in the canopy, has not previously been described for the stay-green introgressions. We found that tiller leaf area rather than transpiration efficiency, or transpiration per leaf area, was the main driver of weekly transpiration and the reduced pre-flowering water use in stay-green lines. In soils with good water-holding capacity, any water savings during the pre-anthesis period increases water availability during the post-anthesis period, therefore allowing plants to retain photosynthetic capacity for longer by “staying green” during grain filling.

1. Introduction

Sorghum is a grain crop that is well adapted to hot and dry climates. For this reason, it is the main summer crop of the rain-fed agricultural production systems of north-eastern Australia, where it is an important rotational crop supplying feed grain to the livestock industry (Starasts, 2012). Sorghum is also a staple crop for more than 500 million people in over 30 countries, with particular significance in sub-Saharan Africa and central-western India.

Rainfall is highly variable and sporadic in these areas. However, where soils have good water-holding capacities, the crops can survive on stored subsoil moisture. The combination of dry atmospheric conditions and high average temperatures leads to a particularly large difference in vapour pressure between the saturated leaf cavities and the less than saturated atmosphere (Sadras and McDonald, 2012).

To capture CO_2 for photosynthesis, leaves must open their stomata, but inadvertently lose water via transpiration. The rate of water loss is proportional to the vapour pressure deficit (VPD) of the atmosphere (Bierhuizen and Slatyer, 1965). While VPD drives transpiration through the leaf to the atmosphere, it has little effect on CO_2 uptake. As a consequence, plants grown in hot and dry environments often transpire wastefully.

Water use may be restricted either through shorter leaf area duration (earlier maturity), smaller leaf area, or reduced transpiration rates per leaf area, which in turn is affected by stomatal conductance and hydraulic conductivity of leaves and roots (Brodribb, 2009; Sack et al., 2015).

Apart from reducing total water use, which limits growth, some plants may be more efficient at converting the transpired water into carbon (Tanner and Sinclair, 1983). At the crop level, this efficiency is generally termed water use efficiency (WUE) and is

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defined as biomass divided by water used by the crop. Depending on the definition, biomass can either mean total biomass (including roots), just shoot biomass, or even simply grain yield. Water used may include plant transpiration, soil evaporation, irrigation, rainfall and moisture stored in the soil (Mortlock, 2014; Passioura, 1996; Sadras and McDonald, 2012; Vadez et al., 2014).

The efficiency with which individual plants turn transpired water into biomass, on the other hand, is generally termed transpiration efficiency (TE). It is also the genetic component of WUE (Vadez et al., 2014), which is why it is of particular interest to plant breeders and physiologists.

A third way to adapt to water limitation is to optimise the temporal distribution of transpiration so that water use is shifted to periods that are particularly critical for the formation of grain yield, such as during grain filling. It may be more important to maximise harvest index, and ultimately grain yield, than overall WUE (Rebetzke and Richards, 1999; Sadras and Connor, 1991; Siddique et al., 1990). This is particularly effective on soils that have good water-holding capacity, and any mechanism that conserves stored subsoil moisture for use during grain filling, may be beneficial for yield.

According to a crop modelling study, about 70% of sorghum crops grown in the northern grain belt of Australia are predicted to experience either mild or severe drought stress during the grain-filling period (Chapman et al., 2000), but many of the soils in that region have reasonable water-holding capacity.

Stay-green (*Stg*) is a trait that has been associated with delayed leaf senescence, increased harvest index and improved grain yields of sorghum crops when water is limited towards the end of the growing season (Borrell et al., 2000a,b; Jordan et al., 2012). For this reason, it has been an important trait in the Australian sorghum breeding programme.

At least four major quantitative trait loci (*Stg1-4*) have been found which all individually induce delayed leaf-senescence (Harris et al., 2007). Field studies have shown that the *Stg* loci reduced the size of sorghum crop canopies at anthesis, mainly through reduced tillering and smaller upper leaves, which led to reduced water uptake during the pre-flowering period, and therefore increased water availability during the post-flowering period (Borrell et al., 2014a,b).

A similar association between leaf area at flowering, daily water use before flowering and grain yield was also observed in a recent study with soybean (He et al., 2017).

Most of these previous studies simply reported on the association between reduced canopy size at flowering and grain yield under post-flowering water limitation at the crop level, as it can be difficult to accurately measure the dynamic link between leaf area and plant transpiration excluding soil evaporation. In this study, we used single plants in large sealed pots (50 L) to measure weekly water use via lysimetry (Mortlock, 2014). We used near-isogenic lines with four different stay-green QTL introgressed into RTx7000 (which is a highly senescent line) and RTx7000 as a control to examine the effects of the stay-green QTL on these dynamics. We were able to show a close link between increasing plant leaf area, and weekly water uptake during the pre-anthesis period. Plant green leaf area, in particular tiller green leaf area, and not TE or transpiration per leaf area, was the main driver of pre-anthesis water use. Paradoxically, we also discovered that age-related senescence of leaves before flowering was accelerated in lines with a stay-green introgression. Together, with the previously known mechanisms of smaller upper leaves and reduced tiller leaf area (Borrell et al., 2014a,b), this may contribute to the smaller canopy at flowering of stay-green lines.

2. Materials and methods

2.1. Treatments and experiment design

Two experiments in consecutive years were conducted to study the effects of stay-green introgressions on dynamics of leaf area and water use of sorghum lines. Near-isogenic lines with individual introgressions covering the four main stay-green quantitative trait loci (*Stg1-4* QTL) in a senescent background (RTx7000) and the senescent control (RTx7000) (Table 1) were grown in minilysimeters to measure transpiration (T), transpiration efficiency (TE), and canopy-related traits of each genotype. One genotype was grown per minilysimeter.

2.2. Experimental details

Two lysimeter experiments were conducted at Hermitage Research Facility (28°12′ S, 152°06′ E; 480 m above sea level) in south-east Queensland, Australia, during two consecutive summer seasons. Single plants were grown in mini-lysimeters (50- L pots) to determine water use (T), biomass, and TE. Exp1 was planted on 25 February 2006 and harvested on 24 May 2006 when plants had reached anthesis. Exp2 was planted on 22 February 2007 and harvested on 20 April 2007, as soon as all the plants had fully expanded flag leaves on the main stem.

The mini-lysimeters were arranged in three rows within two ventilated, plastic-covered growth tunnels. Rows were two metres and the pot centres one metre apart from each other, so the plants were basically grown in single plant stands. In Exp1, three replicates were grown in one growth tunnel and four replicates in the other growth tunnel; in Exp2, two replicates were grown in each growth tunnel. Both tunnels were orientated north-south and far enough apart from each other not to cause any shading. Front and sides of the growth tunnel were covered with white knitted shade cloth to allow air flow, while the top was covered with white solar weave (both materials Gale Pacific Pty Ltd, Melbourne, Australia). The solar weave excluded rainfall and transmitted approximately 70% of the incident solar radiation. The lysimeter pots were made from cylindrical PVC tubes, 300 mm diameter and 750 mm high, which were filled with a 3:1:1 mix of alluvial clay soil, loam and feedlot manure. Each plant was fertilised with 30 g of Osmocote Plus (16% N, 3.5% P, 10% K plus trace elements; Scotts Pty Ltd, Baulkham Hills, Australia).

Temperature and humidity sensors (Tinytag, Gemini Data Loggers Ltd, Chichester, UK) were installed inside each growth tunnel, and hourly and daily maximum and minimum temperatures and relative humidity were recorded. Maximum daily vapour pressure deficits for each growth tunnel were determined from the hourly temperature and humidity records (Rosenberg et al., 1983).

2.3. Water use measurements

The pots were fully saturated with water and drained to field capacity before the basal drainage hole was sealed. One plant per

Table 1
Genotypes grown in lysimeter experiments.

Genotype	*Stg* QTL	Description
RTx7000	RTx7000	Senescent hybrid
NIL 6078-1	*Stg*1	Entire *Stg1* QTL
NIL 2219-3	*Stg*2	Entire *Stg2* QTL
NIL 2290-19	*Stg*3	Entire *Stg3* QTL
NIL 6085-9	*Stg*4	Entire *Stg4* QTL

mini-lysimeter was grown. Black plastic sheeting was placed around the base of the plant to cover the soil surface, eliminating soil evaporation. Lysimeters were weighed weekly. A starting weight was recorded for each pot at field capacity. A control pot filled with soil, watered to field capacity and sealed exactly like the treatment pots, but without a plant grown in it, was also included in each replicate to check for any water loss from the system itself. Since the control pots did not change in weight from week to week, the water loss recorded for the experimental lysimeters was taken as water transpired by the plants. The amount of water used was replaced weekly by watering the pot back to its starting weight. Water use measurements were taken over a 56 and 37 - day period in Exp1 and Exp2, respectively. Weekly water use data was normalised for VPD by dividing by the average of the daily maximum VPD for that week and that particular growth tunnel.

2.4. Leaf measurements

Detailed leaf observations were made on four replicates in each experiment. Fully expanded and senesced leaf number was recorded at weekly intervals for both main culms and tillers as described by Hammer et al. (1993). A leaf was considered fully expanded when its ligule became visible above the enclosing sheath of the previous leaf. A leaf was considered senesced when more than 50% of its area turned yellow. Leaf area of each individual fully expanded leaf was estimated non-destructively from the product of its length, greatest width, and a shape factor of 0.57, which was established by regressing the product of width and length of a leaf against its actual leaf area measured destructively at the end of the experiment. These estimates of individual leaf sizes, combined with weekly observations of fully expanded and senesced leaves, allowed the estimation of weekly green leaf area (Muchow and Carberry, 1990). At the time of the destructive harvest at the end of the experiment, leaf area of every remaining green leaf was measured with an electronic planimeter (DIAS image analysis system, Delta-T Devices Ltd., Cambridge, UK).

Leaf appearance rate (LAR; leaves per °C day) was calculated by dividing final leaf number by thermal time to full extension of the final leaf.

2.5. Biomass harvests

One replicate in Exp1 was harvested at the beginning of the weekly water use measurements to determine starting biomass per plant. As biomass at that time was only around 4 g per plant, which equates to less than 3% of the final biomass, starting biomass was regarded as negligible. The other six replicates in Exp1 were harvested 88 days after sowing, corresponding to about ten days after anthesis. Anthesis was defined as the time when 50% of the florets on the main-stem panicle were flowering. In Exp2, plants were harvested 57 days after sowing which was about two weeks before anthesis, due to imminent cooler weather and risk of frosts. Above-ground plant material was separated into main stems and tillers and then divided into stem (including panicles and leaf sheaths), green leaf and dead leaf portions. A leaf was considered green when at least 50% of its area was still green. For the four detailed replicates, green leaves were treated individually to establish leaf area and weight of each leaf. For the remaining two replicates, leaves were bulked and total plant green leaf area and biomass were established only. All biomass samples were dried in a forced-draught oven at 80 °C for three to four days before weighing.

2.6. Data analysis

Experiments with completely randomised block designs were planted in each year with five genotypes (a senescent control and four *Stg* QTL lines). Data from both years were combined to test for effects of genotype and experiment and their interactions (Table 3). The general form of the mixed model is:

$$y = X\beta + Z\mu + e$$

where the response (vector y) is modelled by a set of fixed effects (vector β) and random effects (vector μ and vector ε). The design matrices X and Z assign the fixed and random effects respectively to the observations (Smith et al., 2001).

A mixed model was fitted with year and genotype as fixed effects and blocks within years as random, using the lme4 package in R (Bates et al., 2015). The linear mixed model applied fixed effects for year, genotype and year by genotype interaction and

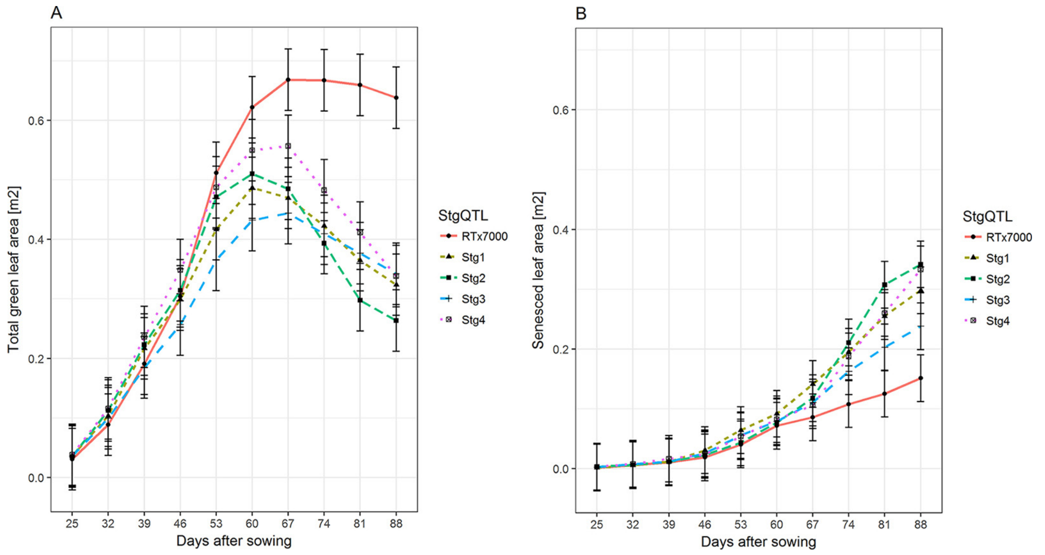

Fig. 1. Dynamics of total green (A) and senesced (B) leaf area per plant [m^2] for the entire duration of Experiment 1 and all genotypes (RTx7000: senescent, no stay-green QTL; *Stg1*: stay-green QTL 1; *Stg2*: stay-green QTL 2; *Stg3*: stay-green QTL 3; *Stg4*: stay-green QTL 4). Points are least squares means for weekly leaf area calculations predicted by the linear mixed model. Averaged across all genotypes, flag leaves were fully expanded by 57 days after sowing and plants reached anthesis by 78 days after sowing.

random effects for trial design aspects of replicates and residuals. Since we were especially interested in the senescent control compared with the remaining lines we fitted an orthogonal contrast to test the senescent control (RTx7000) compared to the four *Stg* QTL lines.

Significance levels for the fixed effects were determined using ANOVA. All analyses were conducted using the statistical software R (R Core Team, 2016).

For examining the traits by days after sowing we fitted an additional random effect for lysimeter (pot) within block.

Regression analyses were performed to find the relationships of various variables. ANCOVA was used to test whether these relationships differed for specific groups (e.g. experiments or genotypes).

Variates were log transformed, scaled and centred before performing principal component analysis and biplots were visualised using the ggbiplot R package (Vu, 2011).

Most plots were drawn using the ggplot2 package (Wickham, 2016).

3. Results

3.1. Both reduced tiller leaf area and earlier onset of leaf senescence led to smaller green leaf areas per plant in stay-green lines in Experiment 1

Up to about 46 days after sowing (DAS) green leaf area per plant was very similar in all lines in Exp1. However, after 46 DAS green leaf area per plant increased at a faster rate, and for at least a week longer, in the senescent line RTx7000 than in the lines with stay-green QTL (Fig. 1A).

This was not due to differences in leaf number or timing to flag leaf between the lines (Table 3). Instead, it was due to slightly greater main-stem green leaf area growth (Fig. 2A), but particularly greater tiller green leaf area growth in RTx7000 (Fig. 2B). Apart from growing less new leaf area, the stay-green lines senesced older leaves at the bottom of the plants at a much faster rate than RTx7000 (Fig. 1B), despite plants being fully watered and still in the vegetative growth phase.

By the time plants reached anthesis in Exp1 (75–80 DAS for all genotypes), these two effects combined led to much smaller green leaf areas per plant in stay-green lines compared with RTx7000.

3.2. Close link between pre-flowering leaf area dynamics and weekly water use

Weekly water use adjusted for differences in vapour pressure deficit increased linearly until around 60 DAS which corresponded with the time of linear leaf area growth (Fig. 3). Leaf area was a strong driver for water use, reflected in a strong positive correlation between total green leaf area per plant and weekly transpiration per plant adjusted for daily maximum VPD for the days before 60 DAS ($y = 8.85x - 0.29$, $n = 80$, $R^2 = 0.81$, $p < 0.001$). Analysis of covariance showed that there were no significant differences in this relationship between genotypes (Fig. 4).

A linear correlation, albeit with significantly different slope and intercept was also observed between total green leaf area per plant and weekly transpiration adjusted for VPD between 28 and 54 DAS in Experiment 2 ($y = 5.16x - 0.03$, $n = 100$, $R^2 = 0.75$, $p < 0.001$).

Between 60 DAS and the end of Experiment 1 (60–88 DAS), the relationship was weaker ($y = 10.04x - 0.58$, $n = 120$, $R^2 = 0.66$, $p < 0.001$). This was mainly due to a general decline in both leaf area and transpiration in most genotypes, with leaf area remaining stable and transpiration declining in the RTx7000 genotype (Fig. 3). The decline in total green leaf area per plant in the lines with a *Stg* QTL was driven by increased leaf senescence (Fig. 1).

3.3. Tiller green leaf area was the main driver for weekly water use within each experiment

Total green leaf area per plant was largely determined by tiller green leaf area (Fig. 5). There was a significant effect of *Stg* QTL on final green leaf area per plant ($p < 0.01$), which was driven by significant differences ($p < 0.01$) in tiller green leaf area. The

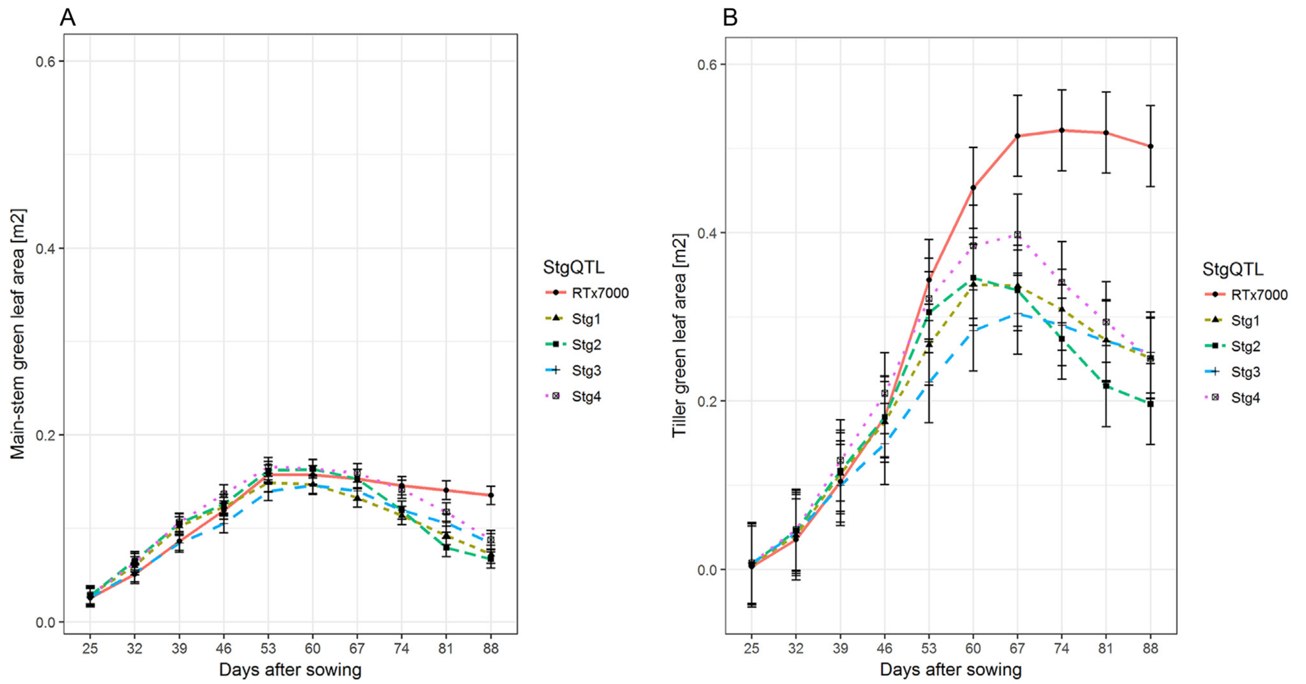

Fig. 2. Dynamics of main-stem (A) and tiller (B) green leaf area per plant [m^2] for the entire duration of Experiment 1 and all genotypes. (RTx7000: senescent, no stay-green QTL; *Stg1*: stay-green QTL 1; *Stg2*: stay-green QTL 2; *Stg3*: stay-green QTL 3; *Stg4*: stay-green QTL 4). Points are least squares means for weekly leaf area calculations predicted by the linear mixed model. Averaged across all genotypes, flag leaves were fully expanded by 57 days after sowing (DAS) and plants reached anthesis by 78 days after sowing.

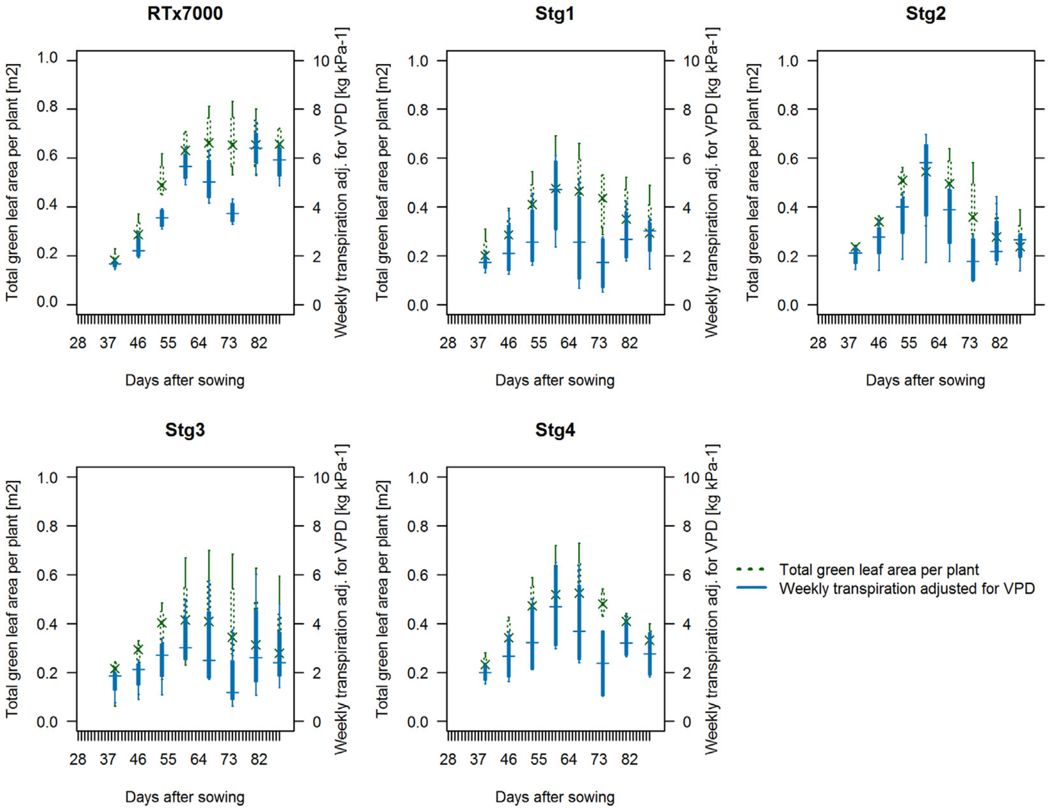

Fig. 3. Box plots for weekly total leaf area per plant [m^2] versus weekly transpiration adjusted for average maximum VPD (kg kPa^{-1}) for all genotypes (RTx7000: senescent, no stay-green QTL; *Stg1*: stay-green QTL 1; *Stg2*: stay-green QTL 2; *Stg3*: stay-green QTL 3; *Stg4*: stay-green QTL 4). Points are averages of four replicates. Averaged across all genotypes, flag leaves were fully expanded by 57 days after sowing and plants reached anthesis by 78 days after sowing.

contrast between the senescent control, RTx7000, and lines with stay-green QTL grouped together was significant for both final green leaf area and tiller green leaf area ($p < 0.001$ and 0.01, respectively) (Table 3).

As total green leaf area per plant was largely determined by tiller green leaf area, there was a strong positive relationship between tiller green leaf area and transpiration during plant growth in Exp1. A similar, albeit weaker relationship, existed in Exp2 (Fig. 6).

Final plant green leaf areas were much greater in Exp2 than in Exp1 ($1.10 \pm 0.04\,m^2$ versus $0.50 \pm 0.03\,m^2$, $p < 0.001$) (Table 3). This increase in plant leaf area was due to greater final leaf number (18.3 leaves versus 15.5 leaves in Exp2 and Exp1, respectively), which in turn was due to greater leaf appearance rate (0.037 versus 0.031 leaves per °C day) in Exp2.

Tiller numbers, on the other hand, were generally lower in Exp2 compared with Exp1 (4.70 ± 0.70 versus 6.80 ± 0.70) (Table 3). Also, tiller leaf areas and consequently total plant green leaf areas, were more similar between genotypes in Exp2 compared with Exp1, where the range of tiller green leaf areas among genotypes was much greater.

3.4. Differences in plant and tiller green leaf areas also led to differences in cumulative water use

While cumulative water use was similar for all genotypes during the first few weeks of Exp1, after about 74 DAS the senescent line RTx7000 had much greater cumulative water use (not adjusted for VPD) compared with lines with stay-green QTL (Fig. 7A). This corresponded with the time when plant leaf areas (and tiller green leaf areas) were most different between RTx7000 and the stay-green lines. In Exp2, where plant and tiller green leaf areas were similar, differences in cumulative water use between the lines were smaller and not significant (Fig. 7B).

Similarly, total water use per plant at the time of harvest was strongly correlated with final tiller green leaf area per plant in Exp1 ($y = 45.9x + 8.09$, $n = 30$, $R^2 = 0.73$, $p < 0.001$), but the relationship was weaker in Exp2 ($y = 20.1x + 14.09$, $n = 20$, $R^2 = 0.40$, $p < 0.01$).

3.5. Transpiration efficiency and transpiration per leaf area were not affected by stay-green QTL

There was no genotype effect on transpiration efficiency (TE), and lines with stay-green QTL had similar TE (above-ground biomass per water transpired) to the senescent control RTx7000, whether TE was adjusted for VPD or not (Table 3).

To compare transpiration per leaf area in each experiment, we divided total water use up to 54 DAS adjusted for weekly averages of daily maximum VPD by the leaf area accumulated by that day (Transp_LA_54DAS). Transpiration per leaf area by 54 DAS did not differ among genotypes (Table 3).

3.6. There were large differences between experiments

Principal component analysis (PCA) (Fig. 8) illustrates differences between experiments. For example in Exp2, LAR, main and tiller green leaf area per plant and transpiration up to 54 DAS VPD adjusted were larger than in Exp1, while in Exp1 biomass per unit green leaf area was greater. Traits such as Transp_LA_54DAS and final transpiration were slightly greater in Exp1 compared with Exp2.

TE also differed between experiments ($p < 0.05$, Table 2 and Fig. 8). TE was greater in Exp1 which had lower average monthly VPD compared with Exp2 ($5.27 \pm 0.14\,g\,kg^{-1}$ versus 4.75 ± 0.17 g

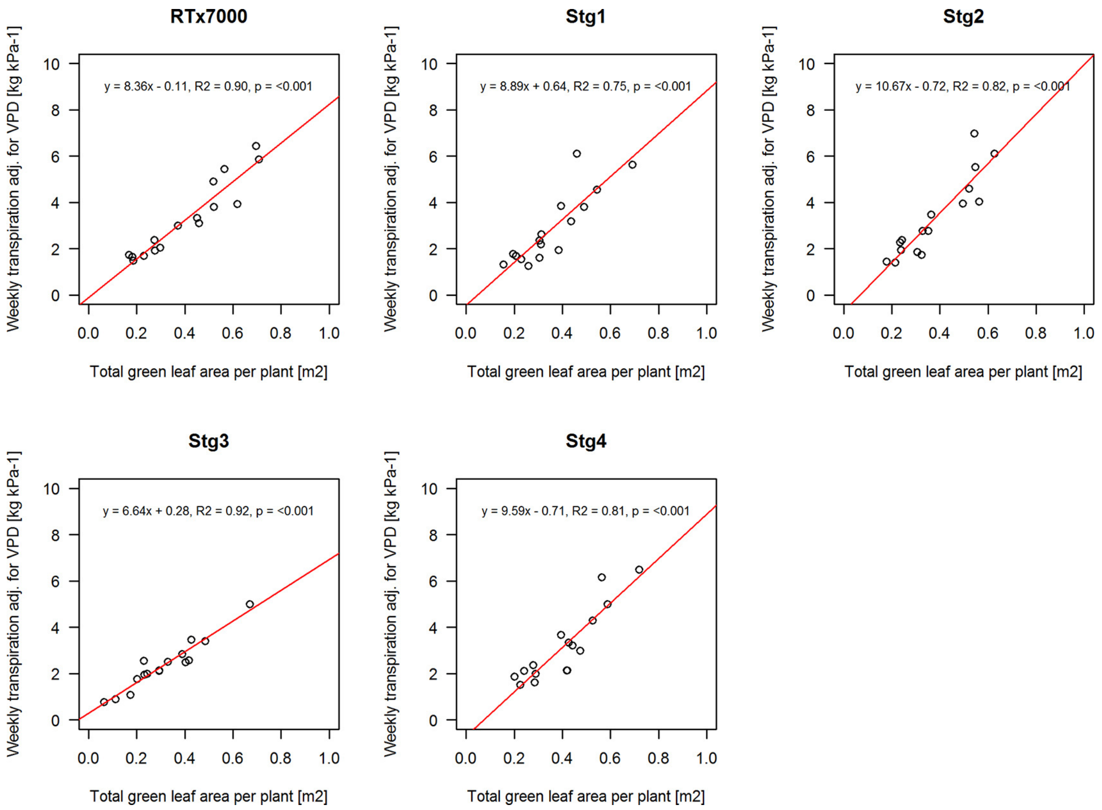

Fig. 4. Weekly transpiration adjusted for average VPD for that week [kg kPa^{-1}] versus total green leaf area per plant [m^2] for each genotype in Exp1 (RTx7000: senescent, no stay-green QTL; *Stg1*: stay-green QTL 1; *Stg2*: stay-green QTL 2; *Stg3*: stay-green QTL 3; *Stg4*: stay-green QTL 4). Data shown are values for all four replicates for each genotype at 39, 46, 53 and 60 days after sowing.

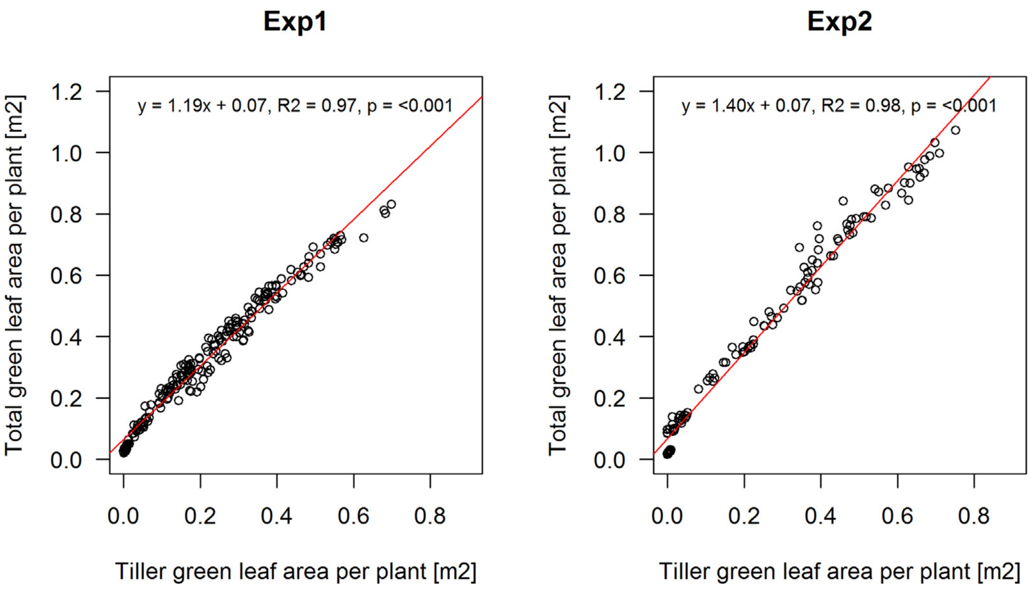

Fig. 5. Total green leaf area per plant [m^2] versus tiller green leaf area per plant [m^2] in both experiments. Data shown are values for all four replicates, five genotypes and dates 25, 32, 39, 46, 53, 60, 67, 74, 81, 88 days after sowing for Experiment 1 (Exp1, left panel) and dates 19, 32, 39, 46, 51 days after sowing for Experiment 2 (Exp2, right panel).

kg^{-1} in Exp1 and Exp2, respectively). Normalising TE by multiplying with daily maximum VPD measured in each of the growth tunnels averaged over the entire experimental season, did not seem to remove differences in TE between experiments (Table 3), but rather increased them. In contrast, transpiration per day and final biomass were similar in both experiments.

The PCA also shows that LAR, main-stem (MGLA_54DAS) and tiller (TGLA_54DAS) green leaf area per plant 54 days after sowing were closely related with cumulative transpiration by 54 DAS. Similarly, final biomass was related to transpiration per day and final TE was correlated with final transpiration, indicating that differences in TE were due more to variation in stomatal conductance than photosynthetic capacity.

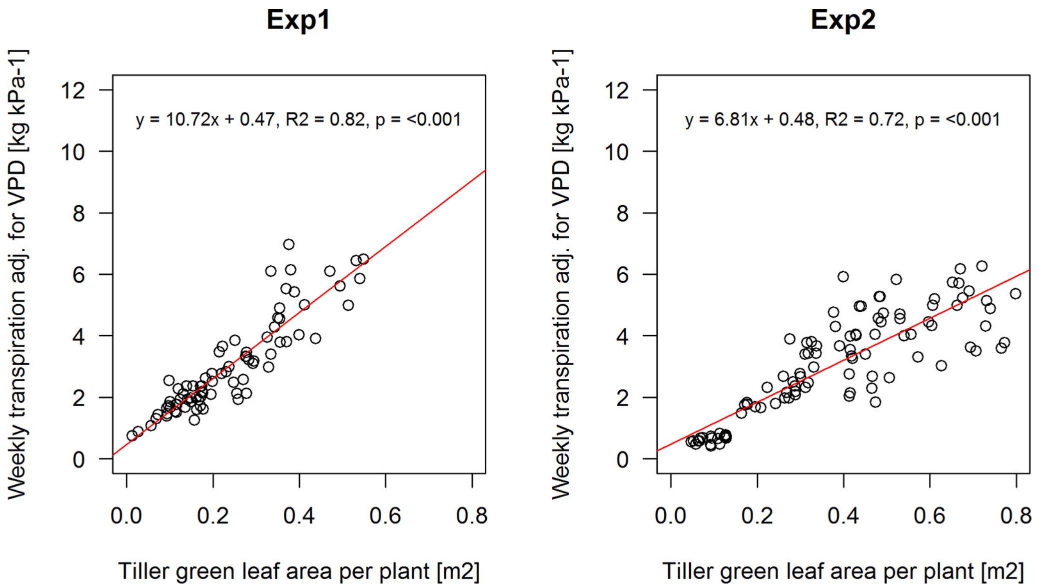

Fig. 6. Weekly transpiration adjusted for average VPD for that week [kg kPa^{-1}] versus tiller green leaf area per plant [m^2] in both Experiment 1 (Exp1, left panel) and Experiment 2 (Exp2, right panel). Data shown are values for all four replicates, five genotypes and dates 39, 46, 53 and 60 days after sowing for Experiment 1 (Exp1) and dates 28, 35, 42, 49, 54 days after sowing for Experiment 2 (Exp2).

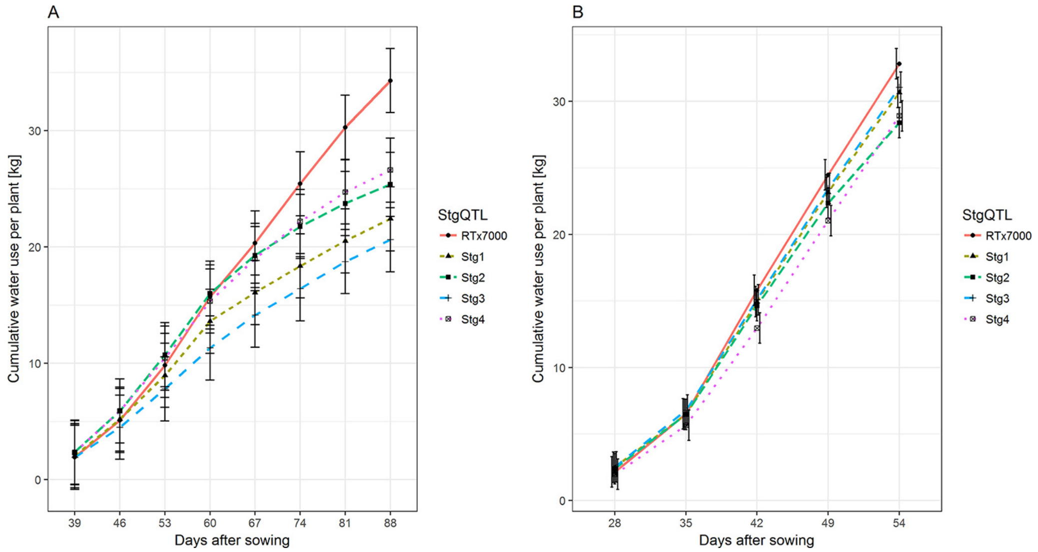

Fig. 7. Cumulative water use per plant [kg] for each genotype in Experiment 1 (A) and Experiment 2 (B) (RTx7000: senescent, no stay-green QTL; *Stg1*: stay-green QTL 1; *Stg2*: stay-green QTL 2; *Stg3*: stay-green QTL 3; *Stg4*: stay-green QTL 4). Points are least squares means for weekly leaf area calculations predicted by the linear mixed model.

Lastly, the PCA illustrates that differences between genotypes were much larger in Exp1 than Exp2.

4. Discussion

Restricting canopy leaf area as a way of reducing crop water use is often discussed in the context of maximising yield under water-limited conditions by conserving water for crop stages that are most critical to yield. Three conditions have to be met for this scenario to work: (a) soils exhibit high water-holding capacity so that water saved in one period is available during a later period, (b) there is no more (or at least very little) water available in the soil profile at maturity (Vadez et al., 2014) and (c) reduced leaf area actually leads to reduced water use (i.e. other factors such as transpiration per leaf area and TE remain unchanged). While many studies have established a link between reduced canopy leaf area at flowering and crop grain yield, and some have compared the water uptake patterns of lines with larger canopies with lines with reduced canopies in a field situation (Borrell et al., 2014b), no study has previously closely looked at the dynamic link between weekly water uptake and leaf area of single plants in large sealed pots so that evaporation from the soil could be excluded.

In the current study, all stay-green QTL (*Stg1-4*) that were introgressed into the RTx7000 background reduced water use, mainly via effects on plant leaf area, while there was no consistent effect on transpiration per leaf area or TE. This is contrary to a previous study that found that some of these stay-green QTL (*Stg1*, *Stg4*) reduced transpiration per leaf area at anthesis in a field situation, while another *Stg* QTL (*Stg2)* increased this trait (Borrell et al., 2014a). Another study assessing *Stg1-4* plus two additional regions (*StgA*, *StgB*), concluded that the effects of these stay-green

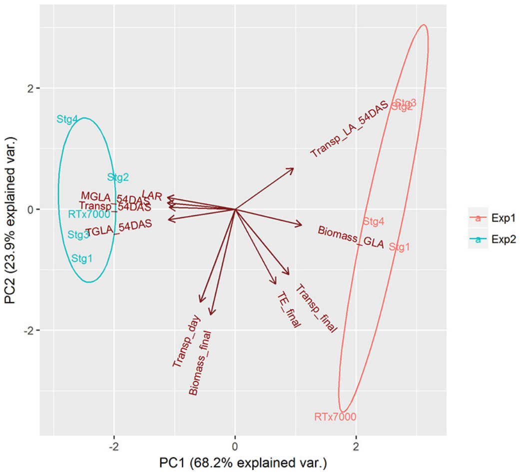

Fig. 8. Bi-plot showing loadings of the variables with principal components 1 and 2 and observations in Exp1 and Exp2. (LAR: leaf appearance rate [leaves per °C day]; MGLA_54DAS: main-stem green leaf area 54 days after sowing [m^2]; TGLA_54DAS: tiller green leaf area 54 days after sowing [m^2]; Transp_54DAS: transpiration by 54 days after sowing adjusted for maximum daily VPD [$kg\,kPa^{-1}$]; Transp_day: transpiration per day adjusted for maximum daily VPD [$kg\,day^{-1}\,kPa^{-1}$]; Biomass_final: final biomass [g]; TE_final: final TE not adjusted for VPD [$g\,kg^{-1}$]; Biomass_GLA: biomass per unit green leaf area [$g\,m^{-2}$]; Transp_LA_54DAS: transpiration per unit green leaf area by 54DAS [$kg\,m^{-2}$], RTx7000: senescent, no stay-green QTL; *Stg1*: stay-green QTL 1; *Stg2*: stay-green QTL 2; *Stg3*: stay-green QTL 3; *Stg4*: stay-green QTL 4).

QTL on TE depended on recipient genetic background (Vadez et al., 2011).

Stg1-4 QTL have previously been shown to reduce tiller number which, in turn, was due to larger cumulative leaf area of leaves 2–9 (Borrell et al., 2014b). Similar increased vigour during early growth, evident in greater leaf appearance rate, has also been associated with a decrease in tiller number in ATx642, which is the donor parent of the *Stg1-4* introgressions, compared to AQL39, a senescent hybrid (van Oosterom et al., 2011). It is thought that competition for carbon between early leaves and tillers lead to this suppression in tillering in genotypes with greater LAR or increased size of early leaves (Alam et al., 2014; Kim et al., 2010). In accordance with this, reducing carbon supply by removing leaves (and therefore photo-assimilate) also led to a reduction in tillering (Kebrom and Mullet, 2015). Contrary to these studies, we found neither a difference in leaf appearance rate (LAR) (Table 3) between the senescent RTx7000 and lines with a stay-green QTL, nor a difference in main-stem leaf area among all genotypes during early leaf area development (Fig. 2A). In fact, tiller green leaf areas were also quite similar until 46 DAS in Exp1 (Fig. 2B), and only after that did they start to separate, with tiller green leaf area increasing more in RTx7000 compared with the stay-green line. In addition, due to senescence of early leaves, tiller green leaf areas (and main-stem leaf areas) started declining from 53 DAS in lines with stay-green QTL, but not in RTx7000. This is the first time, to our knowledge, that this mechanism of controlling total plant green leaf area has been reported in sorghum genotypes with stay-green QTL.

The effect of increased LAR on reduced plant size was previously found to be temperature dependent, such that genotypic differences in tillering were reduced under high temperature, with main-shoot leaf number increasing more in hybrids with greater LAR (van Oosterom et al., 2011). While in our study, differences in LAR did not explain differences in tiller green leaf area within each experiment, we also observed a temperature response when comparing leaf areas between experiments. Higher average temperatures early during Exp2 (Table 2) led to greater final leaf number (18.5 leaves versus 15.7 leaves in Exp2 and Exp1, respectively), which in turn was due to greater leaf appearance rate (0.039 versus 0.030 leaves per °C day) in Exp2 (Table 3). As a consequence, total green leaf areas per plant were more than double in Exp2 compared to Exp1 (Table 3), tiller numbers were generally lower, and genotypic differences in tiller number were greatly diminished.

Hence the effects of stay-green QTL on reducing tiller leaf area might be restricted to cooler environments that are conducive to tillering. However, despite the absence of differences in tiller green leaf area between genotypes in Exp2, there was still a trend (not significant) for lines with stay-green QTL to use less water than the senescent line RTx7000. It is conceivable that differences in leaf area among genotypes are the dominant factor driving T when differences in leaf area are large, but when genotypes exhibit similar leaf areas, leaf level WUE and TE may become more important. In accordance with this hypothesis, there was a trend for TE to be greater in all stay-green lines compared to the senescent line in Exp2. It should also be noted that these experiments were conducted under non-limiting water conditions, and leaf area growth driven by higher temperature is likely restricted by water availability in rain-fed sorghum growing areas. Finally, the stay-green trait has been associated with increased grain yield over a large range of environments (mild to severe post-anthesis drought) that are also typically affected by high temperatures (Jordan et al., 2012).

However, smaller leaves and reduced transpiration rates likely lead to increased canopy temperatures (Sadras and Calderini, 2015). Cooler canopies have been shown to be associated with greater yields in some situations, e.g. where water is limiting but present at depth (Lopes and Reynolds, 2010).

The effects observed here on single plants were small, but these effects will be cumulative across entire canopies. Even seemingly small water savings before flowering can lead to considerable yield advantages at the crop level when these savings are available during grain filling, as a study with wheat has shown where each mm extra of available water during grain fill was predicted to produce 55 kg ha^{-1} more yield (Manschadi et al., 2006). Similarly in sorghum, each mm extra of available water during grain filling was shown in a field experiment to produce an additional 50 kg ha^{-1} of grain yield (Borrell et al., 2014a).

Table 2
Mean daily maximum and minimum temperature, relative humidity (inside the growth tunnel) and solar radiation (outside growth tunnel) during both experiments.

	Mean daily max temp [°C]	Mean daily min temp [°C]	Mean relative humidity [%]	VPD [kPa]	Mean daily solar radiation [$MJ\,m^{-2}$]
2006					
March	28.0	14.6	65.1	1.2	19.7
April	28.0	10.4	63.1	1.2	16.9
May	23.0	3.6	60.3	0.9	15.0
2007					
March	37.6	13.9	59.9	2.4	21.0
April	29.6	11.7	66.3	1.2	16.7
May	23.8	16.9	55.4	1.2	12.6

Table 3
ANOVA results and least squares mean ± standard error of the mean for the most important traits.

	Final green leaf area per plant [m^2]	Main-stem green leaf area [m^2]	Tiller green leaf area [m^2]	Tillers per plant	Final leaf number	LAR [leaves per °C day]	Days to flag leaf	Final biomass per plant [g]	TE [$g\,kg^{-1}$]	TE adjusted for VPD [$g\,kg^{-1}\,kPa^{-1}$]	Total transpiration up to 54 DAS adjusted for VPD [$kg\,kPa^{-1}$]	Green leaf area per plant 54 DAS [m^2]	Transpiration per leaf area up to 54 DAS adjusted for VPD [$kg\,m^{-2}\,kPa^{-1}$]
	p-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value
Exp	<0.001 (***)	<0.01 (**)	<0.001 (***)	0.07 (ns)	<0.001 (**)	<0.001 (**)	<0.001 (**)	0.31 (ns)	<0.05 (*)	<0.01 (**)	<0.01 (**)	<0.01 (**)	0.12 (ns)
Stg QTL	<0.01 (**)	0.31 (ns)	<0.01 (**)	0.84 (ns)	0.23 (ns)	0.62 (ns)	0.25 (ns)	<0.05 (*)	0.10 (ns)	0.06 (ns)	0.65 (ns)	0.71 (ns)	0.70 (ns)
Exp**Stg* QTL	0.28 (ns)	0.83 (ns)	0.28 (ns)	0.71 (ns)	0.39 (ns)	0.59 (ns)	0.59 (ns)	0.09 (ns)	<0.05 (*)	<0.05 (*)	0.14 (ns)	0.26 (ns)	0.56 (ns)
Stg QTL RTx7000 versus Rest:Exp1	<0.001 (***)	0.05 (ns)	<0.01 (**)	0.21 (ns)	0.80 (ns)	0.56 (ns)	0.39 (ns)	<0.01 (**)	0.09 (ns)	0.20 (ns)	0.78 (ns)	0.13 (ns)	0.10 (ns)
Stg QTL RTx7000 versus Rest:Exp2	0.28 (ns)	0.41 (ns)	0.46 (ns)	0.70 (ns)	0.43 (ns)	0.83 (ns)	0.32 (ns)	0.28 (ns)	0.14 (ns)	0.18 (ns)	0.11 (ns)	0.72 (ns)	0.41 (ns)
	est	est	est	est	est	est	est	est	est	est	est	est	est
Exp													
Exp1	0.50 ± 0.03	0.12 ± 0.01	0.38 ± 0.03	6.80 ± 0.70	15.7 ± 0.20	0.030 ± 0.0006	56.8 ± 0.61	133.1 ± 6.66	5.27 ± 0.14	6.40 ± 0.47	7.4 ± 0.81	0.45 ± 0.04	16.3 ± 0.64
Exp2	1.10 ± 0.04	0.31 ± 0.01	0.79 ± 0.03	4.70 ± 0.70	18.5 ± 0.20	0.039 ± 0.0006	53.3 ± 0.61	143.8 ± 7.85	4.75 ± 0.17	9.54 ± 0.57	13.3 ± 0.81	0.92 ± 0.04	14.7 ± 0.64
Stg QTL													
RTx7000	0.93 ± 0.05	0.24 ± 0.01	0.70 ± 0.04	6.38 ± 0.78	17.4 ± 0.31	0.035 ± 0.0009	55.8 ± 0.96	167.63 ± 11.33	5.12 ± 0.18	8.04 ± 0.43	11.0 ± 0.75	0.73 ± 0.04	15.2 ± 0.74
Stg1	0.83 ± 0.05	0.21 ± 0.01	0.62 ± 0.04	5.38 ± 0.78	17.5 ± 0.31	0.035 ± 0.0009	57.5 ± 0.96	150.66 ± 11.78	5.36 ± 0.19	8.54 ± 0.44	10.2 ± 0.75	0.69 ± 0.04	15.2 ± 0.74
Stg2	0.69 ± 0.05	0.20 ± 0.01	0.49 ± 0.04	5.88 ± 0.78	16.8 ± 0.31	0.034 ± 0.0009	54.0 ± 0.96	121.83 ± 11.33	5.02 ± 0.18	8.09 ± 0.43	10.4 ± 0.75	0.66 ± 0.04	16.3 ± 0.74
Stg3	0.83 ± 0.05	0.22 ± 0.01	0.61 ± 0.04	5.63 ± 0.78	17.0 ± 0.31	0.034 ± 0.0009	59.3 ± 0.96	125.44 ± 11.78	4.78 ± 0.19	7.70 ± 0.44	9.8 ± 0.75	0.66 ± 0.04	15.5 ± 0.74
Stg4	0.73 ± 0.05	0.22 ± 0.01	0.52 ± 0.04	5.38 ± 0.78	16.6 ± 0.31	0.035 ± 0.0009	57.5 ± 0.96	126.65 ± 11.33	4.78 ± 0.18	7.46 ± 0.43	10.4 ± 0.75	0.69 ± 0.04	15.2 ± 0.74
*Exp*Stg QTL*													
Exp1													
RTx7000	0.71 ± 0.06	0.14 ± 0.02	0.57 ± 0.06	8.25 ± 1.10	15.8 ± 0.44	0.031 ± 0.0012	55.8 ± 1.36	183.52 ± 14.34	5.60 ± 0.23	6.78 ± 0.55	7.6 ± 1.06	0.51 ± 0.06	15.0 ± 1.04
Stg1	0.49 ± 0.06	0.10 ± 0.02	0.39 ± 0.06	6.00 ± 1.10	15.8 ± 0.44	0.030 ± 0.0012	57.5 ± 1.36	145.00 ± 15.70	5.58 ± 0.25	6.79 ± 0.57	7.0 ± 1.06	0.42 ± 0.06	16.2 ± 1.04
Stg2	0.34 ± 0.06	0.10 ± 0.02	0.24 ± 0.06	7.00 ± 1.10	15.3 ± 0.44	0.031 ± 0.0012	54.0 ± 1.36	104.87 ± 14.34	5.15 ± 0.23	6.28 ± 0.55	8.3 ± 1.06	0.47 ± 0.06	17.5 ± 1.04
Stg3	0.52 ± 0.06	0.13 ± 0.02	0.39 ± 0.06	6.25 ± 1.10	16.0 ± 0.44	0.030 ± 0.0012	59.2 ± 1.36	99.52 ± 15.70	4.66 ± 0.25	5.61 ± 0.57	6.1 ± 1.06	0.37 ± 0.06	16.5 ± 1.04
Stg4	0.46 ± 0.06	0.12 ± 0.02	0.34 ± 0.06	6.25 ± 1.10	15.5 ± 0.44	0.030 ± 0.0012	57.5 ± 1.36	132.75 ± 14.34	5.37 ± 0.23	6.55 ± 0.55	8.1 ± 1.06	0.49 ± 0.06	16.2 ± 1.04
Exp2													
RTx7000	1.16 ± 0.07	0.33 ± 0.02	0.83 ± 0.07	4.50 ± 1.10	19.0 ± 0.44	0.040 ± 0.0012	54.0 ± 1.36	151.74 ± 17.56	4.63 ± 0.28	9.30 ± 0.67	14.4 ± 1.06	0.94 ± 0.06	15.4 ± 1.04
Stg1	1.18 ± 0.07	0.32 ± 0.02	0.86 ± 0.07	4.75 ± 1.10	19.3 ± 0.44	0.040 ± 0.0012	54.0 ± 1.36	156.32 ± 17.56	5.13 ± 0.28	10.30 ± 0.67	13.5 ± 1.06	0.96 ± 0.06	14.2 ± 1.04
Stg2	1.04 ± 0.07	0.30 ± 0.02	0.74 ± 0.07	4.75 ± 1.10	18.3 ± 0.44	0.038 ± 0.0012	54.0 ± 1.36	138.80 ± 17.56	4.90 ± 0.28	9.90 ± 0.67	12.5 ± 1.06	0.84 ± 0.06	15.1 ± 1.04
Stg3	1.13 ± 0.07	0.31 ± 0.02	0.82 ± 0.07	5.00 ± 1.10	18.0 ± 0.44	0.038 ± 0.0012	54.0 ± 1.36	151.37 ± 17.56	4.89 ± 0.28	9.80 ± 0.67	13.6 ± 1.06	0.95 ± 0.06	14.5 ± 1.04
Stg4	1.01 ± 0.07	0.31 ± 0.02	0.69 ± 0.07	4.50 ± 1.10	17.8 ± 0.44	0.040 ± 0.0012	50.2 ± 1.36	120.55 ± 17.56	4.19 ± 0.28	8.38 ± 0.67	12.7 ± 1.06	0.90 ± 0.06	14.1 ± 1.04

ns – not significant at $\alpha = 0.05$.

Stay-green is an integrated drought adaptation trait characterised by a distinct green leaf phenotype during grain filling under terminal drought. The phenotype, generally exhibited late in the grain filling period, is an emergent consequence of processes occurring before anthesis, such as decreased tillering and smaller upper leaves, which ultimately result in a smaller canopy size at anthesis. This reduction in transpirational leaf area conserves soil water before anthesis for subsequent use during grain filling.

However, a stay-green phenotype was not observed in the experiments reported in this paper. There are a number of reasons for this. First, the experiments were fully irrigated, i.e. the plants did not experience water deficits that would induce drought-related senescence. Second, the experiments were terminated either two weeks before anthesis (Exp2) or 10 days after anthesis (Exp1), enabling only pre-anthesis processes to be evaluated. As expected, the lines with stay-green QTL in this study exhibited less green leaf area around anthesis compared with the senescent control, particularly in Experiment 1, largely due to reduced tiller green leaf area (Fig. 2B). While the rapid senescence of older, lower leaves in the stay-green lines before anthesis seems to contradict the “stay-green” concept, this novel mechanism is simply another way for the plant to conserve water before anthesis, thereby increasing the availability of water for grain filling. The emergent consequence of this should, in fact, be a stay-green phenotype.

While stay-green QTL reduced plant leaf area leading to pre-flowering water savings, this also reduced plant biomass, which may have negative effects on grain yield in situations where water is not limiting. However, Borrell et al. (2014b) found no consistent yield penalty associated with stay-green QTL in an irrigated control. Reduced early vigour may also increase soil evaporation. However, on soils where rainfall is stored in the subsoil while the top layers are usually dry, and most of the rainfall is received in storm events that are typically sporadic but substantial, evaporation is much less important.

VPD has a direct effect on transpiration (Bierhuizen and Slatyer, 1965), so to adjust for this, we have divided weekly T by the average of daily maximum VPD (Tanner and Sinclair, 1983). However, normalising T in this way to compare between two seasons often results in significant differences in TE (Tolk and Howell, 2009), as was the case in our study, too. This is thought to be due to the errors induced by averaging VPD over an entire season. Another reason for the difference in TE adjusted for VPD between our two experiments might be that plants were harvested about ten days after anthesis in Exp1, but two weeks before anthesis in Exp2. It has previously been noted that studies with shorter duration generally report greater TE than longer studies (Tolk and Howell, 2009). TE adjusted for VPD for both our experiments falls well within the range of TE reported elsewhere, e.g. $6.3\,\mathrm{g\,kg^{-1}\,kPa^{-1}}$ (Tolk and Howell, 2009), $9\,\mathrm{g\,kg^{-1}\,kPa^{-1}}$ (Mortlock and Hammer, 2000) and $11.3\,\mathrm{g\,kg^{-1}\,kPa^{-1}}$ (Steduto and Albrizio, 2005).

Tillering is an important trait in sorghum that contributes considerably to grain yield and gives the crop some plasticity, both spatially by allowing it to adapt to different population densities, but also temporally by providing some insurance against total yield losses in the case of adverse environmental effects, such as heat events, that affect yield formation on the main head. But tillers can come at a cost. As we have shown here, tiller leaf area is tightly linked with plant water use. By restricting their leaf area growth, and even shedding some leaf area early during the vegetative growth phase via senescence, sorghum lines with stay-green introgressions use soil moisture more slowly, effectively shifting water use to the later crop stages. This ensures that they remain productive for longer during the critical grain-filling period. The fact that stay-green has been so important in breeding sorghum hybrids with increased tolerance to post-anthesis drought in Australia is testament that this mechanism works well in the field.

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