ORIGINAL PAPER

Studying the genetic basis of drought tolerance in sorghum by managed stress trials and adjustments for phenological and plant height differences

P. K. Sabadin · M. Malosetti · M. P. Boer · F. D. Tardin · F. G. Santos · C. T. Guimarães · R. L. Gomide ·
C. L. T. Andrade · P. E. P. Albuquerque · F. F. Caniato · M. Mollinari · G. R. A. Margarido · B. F. Oliveira ·
R. E. Schaffert · A. A. F. Garcia · F. A. van Eeuwijk · J. V. Magalhaes

Abstract Managed environments in the form of well watered and water stressed trials were performed to study the genetic basis of grain yield and stay green in sorghum with the objective of validating previously detected QTL. As variations in phenology and plant height may influence QTL detection for the target traits, QTL for flowering time and plant height were introduced as cofactors in QTL analyses for yield and stay green. All but one of the flowering time QTL were detected near yield and stay green QTL. Similar co-localization was observed for two plant height QTL. QTL analysis for yield, using flowering time/plant height cofactors, led to yield QTL on chromosomes 2, 3, 6, 8 and 10. For stay green, QTL on chromosomes 3, 4, 8 and 10 were not related to differences in flowering time/plant height. The physical positions for markers in QTL regions projected on the sorghum genome suggest that the previously detected plant height QTL, *Sb-HT9-1*, and *Dw2*, in addition to the maturity gene, *Ma5*, had a major confounding impact on the expression of yield and stay green QTL. Co-localization between an apparently novel stay green QTL and a yield QTL on chromosome 3 suggests there is potential for indirect selection based on stay green to improve drought tolerance in sorghum. Our QTL study was carried out with a moderately sized population and spanned a limited geographic range, but still the results strongly emphasize the necessity of corrections for phenology in QTL mapping for drought tolerance traits in sorghum.

P. K. Sabadin · F. D. Tardin · F. G. Santos ·
C. T. Guimarães · R. L. Gomide · C. L. T. Andrade ·
P. E. P. Albuquerque · F. F. Caniato · B. F. Oliveira ·
R. E. Schaffert · J. V. Magalhaes (✉)
Embrapa Maize and Sorghum, Rod. MG 424,
Km 65, Sete Lagoas, MG 35701-970, Brazil
e-mail: jurandir@cnpms.embrapa.br

M. Malosetti · M. P. Boer · F. A. van Eeuwijk
Biometris, Wageningen University, PO. Box 100,
Wageningen, The Netherlands

M. Mollinari · G. R. A. Margarido · A. A. F. Garcia
Department of Genetics, Luiz de Queiroz College of Agriculture,
Universidade de São Paulo, PO. Box 83, Piracicaba,
SP 13400-970, Brazil

B. F. Oliveira
Departmento de Bioengenharia, Universidade Federal de São
João del-Rei, Praça Frei Orlando 170, São João del-Rei,
MG 36307-352, Brazil

Introduction

Drought stress is a serious agronomic problem contributing to severe yield losses worldwide. This agricultural constraint may nevertheless be addressed by developing crops that are well adapted to drought prone environments. Drought tolerance depends on the plant developmental stage at the onset of the stress syndrome, which in sorghum may happen during the early vegetative seedling stage, during panicle development and in post-flowering, in the period between grain filling and physiological maturity (Rosenow and Clark 1995; Rosenow et al. 1996). In particular, post-flowering drought stress can result in significant reductions in crop yield (Rosenow and Clark 1995; Rosenow et al. 1996). Sorghum is one of the most drought tolerant crop species and is an important model system for studying physiological and molecular mechanisms

underlying drought tolerance (Doggett 1988; Ludlow and Muchow 1990; Mullet et al. 2001; Sanchez et al. 2002). Post-flowering drought adaptation in sorghum is associated with the stay green phenotype, which is characterized by the maintenance of green stems and upper leaves under water limitation after flowering (Subudhi et al. 2000).

Several stay green QTL associated with post-flowering drought tolerance have been mapped (Tuinstra et al. 1997, 1998; Crasta et al. 1999; Tao et al. 2000; Xu et al. 2000; Subudhi et al. 2000; Kebede et al. 2001; Haussmann et al. 2002; Sanchez et al. 2002) and molecular markers linked to these QTL are thus available (Hash et al. 2003; Harris et al. 2007; Kassahun et al. 2009). The most common source of stay green has historically been the sorghum line, BTx642 (formerly called B35), a member in the durra race. These studies identified four major *Stay green* QTL designated as *Stg1*, *Stg2*, *Stg3* and *Stg4* as well as many additional minor QTL. *Stg1* and *Stg2* were mapped to sorghum chromosome 3, explaining approximately 20 and 30% of the phenotypic variance, respectively (Xu et al. 2000; Sanchez et al. 2002; Harris et al. 2007). *Stg3* is located on chromosome 2 and *Stg4* on 5, accounting for 16 and 10% of the phenotypic variance, respectively (Sanchez et al. 2002; Harris et al. 2007). These four major QTL were consistently identified in a range of different environments (Subudhi et al. 2000; Tao et al. 2000) and in different genetic backgrounds (Subudhi et al. 2000). In addition, transgressive segregation (Haussmann et al. 2002) and epistatic interactions involving stay green QTL (Subudhi et al. 2000) have both been reported. Epistatic interactions should be considered in breeding programs, since the combination of positive alleles at *Stg2* and *Stg3* was found to explain almost half of the phenotypic variance for the trait, which exceeds the sum of the individual QTL effects (Subudhi et al. 2000).

A positive impact of stay green on grain yield under terminal drought has been observed (Borrell et al. 2000; Jordan et al. 2003; Kassahun et al. 2009). Tuinstra et al. (1997) reported co-localization of stay green and grain yield QTL under drought stress, suggesting that the gene(s) underlying stay green may also result in enhanced yield performance under drought stress. Tuinstra et al. (1998), using near-isogenic lines, found positive associations between these two traits reinforcing the potential for indirect selection based on stay green for improving grain yield under drought stress in sorghum.

Mixed model approaches in QTL mapping allow for modeling heterogeneous genetic variances and correlations between environments and for the adoption of trial-specific structures of the residual genetic variation in multiple environment trials (Boer et al. 2007). Consequently, they are particularly suitable to modeling complex phenotypic responses across environments, including commonly observed genotype by environment interactions (GEI). The

use of mixed models in QTL mapping results in more reliable and realistic estimates of genotypic effects. Simulation studies indicated greater power of mixed model QTL procedures for detecting QTL by environment interactions (QEI) than fixed models would have (Piepho 2005). This is important for drought stress trials for which it is known that substantial and complex GEI occurs (Clarke et al. 1992). In barley, mixed models were used to study QTL in a multi-environment context, which allowed for QTL detection based on environment specific rather than on main effects (Malosetti et al. 2004). The majority of the QTL detected by Boer et al. (2007) in maize, using mixed models, showed significant QEI, indicating that approaches that concentrate on main effect QTL only may produce inferences that are of limited practical use in plant breeding.

According to Pinto et al. (2010), breeding for drought adaptation has been strongly affected by drought escape based on development, whereby sensitive development stages do no coincide with the stress peak. For instance, flowering time tends to be associated with yield (Ludlow and Muchow 1990) but in a rather unpredictable manner. Accordingly, early flowering may be advantageous if it enables a cultivar to escape drought during the reproductive stages whereas late flowering may be beneficial in the cases where drought stress occurs early in the season. In QTL mapping for drought tolerance, unsynchronized phenology may result in the detection of escape-related QTL, which arise mostly from variations in phenology (Pinto et al. 2010), translating into co-localization between phenology QTL and those for yield and stay green. In addition, because other relevant QTL may be missed, QTL with limited practical relevance for drought tolerance breeding may be detected if the confounding effect of phenology is disregarded.

For our study, we adopted a design including water stress and control treatments to study the influence of flowering time on QTL mapping for grain yield and stay green, particularly seeking to validate previously detected QTL for both traits. In addition, we investigated a possible association between plant height and yield QTL. We used a population of moderate size as well as a limited geographical range for the environments, but nevertheless our results strongly indicate that for practically useful conclusions on grain yield and stay green QTL, variations in phenology and plant height should be accounted for in the QTL mapping procedure.

Materials and methods

Field data

A recombinant inbred line (RIL) population was derived from a cross between BR007, a breeding line from the

Embrapa Maize and Sorghum program, and SC283, a sorghum converted line belonging to the guinea race. Both lines showed intermediate tolerance to drought stress in two different trials in Brazil but yield reduction caused by drought stress in SC283 tended to be consistently smaller than in BR007. F_1 plants were self-pollinated and individual F_2 plants were advanced to $F_{7:8}$ by single seed descent (Johnson and Bernard 1962). In 2006 and 2007, ninety RILs were grown under two water regimes in Janaúba, Minas Gerais State, Brazil, at 15°45′20″ Latitude South and 43°16′55″ Longitude West, and 535.370 m altitude. Combinations of years and water regimes were designated environments and the following coding system was adopted in this study: fully irrigated treatments during the whole crop cycle were coded *well watered* (WW) and the post-flowering drought stress treatments were coded *water stress* (WS), with WW or WS being followed by the last two digits of the year in which each trial was conducted.

The experiments were performed in a dark-red latosol, during winter time, from June to October in both years. The annual average of the air temperatures and relative humidity in Janauba are 24.7°C and 65%, respectively and the strongest water deficiency, exceeding 70 mm, is verified between July and October. The 2006 and 2007 trials were thus established in the second and first week of June, respectively. Irrigation was applied twice a week with a conventional sprinkler scheme set to operate at 12 m × 12 m (spacing among sprinklers) and 17 mm h^{-1} of water. Well watered conditions were ensured by applying water to completely replace water loss based on the crop evapotranspiration rate. This was determined based on local climatic data obtained from an automatic weather station by means of the modified Penman–Monteith equation. Water stress was achieved based on the soil water retention curve by interrupting irrigation at 12 days before flowering (considering the average maturity of the trial) in both years to allow for soil moisture depletion to a water stress condition in post-flowering.

Each plot consisted of two 5 m rows, with 0.50 m between rows and 12 plants m^{-1}. The experiments were conducted in a randomized complete blocks design with three replicates in 2006 and in an alpha lattice design with three replicates and 10 incomplete blocks in 2007. Fertilization consisted of 250 kg ha^{-1} of 8-28-16 (NPK) at sowing and 160 kg ha^{-1} of urea applied 30 days after sowing. The plots were organized in a two-dimensional array of 6 rows and 50 columns consisting of 3 replications of 100 genotypes per rep. These 100 genotypes included 90 RILs and the two parents, BR007 and SC283, with SC283 being repeated nine times. Stay green was visually scored based on the percentage of senescent leaves measured at 45 days after flowering (considering the average maturity of the trial) and grain yield was measured in t ha^{-1}. For

phenotypic analysis and QTL mapping, stay green was transformed in green leaf percentage. Diagnostic plots for residuals were inspected and showed no violations of standard ANOVA assumptions, like homoscedasticity and normality. Phenotypic assessments of stay green were performed only under drought stress conditions in both years as variation for this trait in well watered environments was found to be negligible. Plant height data was recorded as the average distance from the soil surface to the tip of the panicle. Flowering time was assessed as the number of days from emergence to 50% flowering (i.e., time at which at least 50% of the plants within the plot have 50% of open flowers).

Statistical analysis of phenotypic data

For each environment, first a model with genotype and block terms taken as random was fit to grain yield and stay green data to obtain estimates for variance components that provide insights into the magnitude of the different sources of variation. For reasons of convenience and computational stability, in subsequent analyses, complete blocks/replicates were considered a fixed effect. For the 2006 randomized complete blocks trial, the following model was fitted as initial model (random terms are underlined in all models presented henceforth), $\underline{y}_{ij} = \mu + \underline{G}_i + r_j + \underline{e}_{ij}$, where $\underline{y}_{ij}$ is the response for genotype i ($i = 1\ldots90$) in replicate j ($j = 1\ldots3$); μ is the general mean; $\underline{G}_i$ is the random genotypic main effect with $\underline{G}_i \sim N(0, \sigma_g^2)$, r_j is the fixed block effect and $\underline{e}_{ij}$ the residual term with $\underline{e}_{ij} \sim N(0, \sigma_e^2)$.

The model adopted for the alpha design in the 2007 trial was $\underline{y}_{ij} = \mu + \underline{G}_i + r_j + \underline{b}_{k(j)} + \underline{e}_{ij}$, where $\underline{b}_{k(j)}$ is the random block k ($k = 1\ldots10$) effect within replicate, with $\underline{b}_{k(j)} \sim N(0, \sigma_b^2)$ and $\underline{e}_{ij}$ is the residual term with $\underline{e}_{ij} \sim N(0, \sigma_e^2)$.

Heritability (h^2) was estimated according to the general formula proposed by Cullis et al. (2006) and Oakey et al. (2006) as $h^2 = 1 - \frac{\text{PEV}}{2 \times \sigma_g^2}$, where PEV is the predicted error variance of genotypic effects and σ_g^2 being the genotypic variance.

A multi-environment mixed model analysis was then performed considering random genetic effects normally distributed with $N(0, G)$, with G being the genetic variance–covariance (VCOV) matrix based on the unstructured model where a specific genetic variance was fitted for each environment and a specific covariance fitted for each pair of environments. The general model was defined as $\underline{y}_{ij(l)} = \mu + r_{j(l)} + E_l + \underline{G}_{il} + \underline{e}_{ij(l)}$, where E_l is the environmental main effect l ($l = 1\ldots4$), $\underline{G}_{il}$ represents the

genotypic main effect together with the GEI for genotype i in environment l and $e_{ij(l)}$ is the genotype by block (replicate) interaction within environment whose variance is allowed to vary with the environment. This general model was further extended to accommodate the complete and incomplete block designs used in 2006 and 2007, respectively. Genetic correlations were estimated by fitting the VCOV for the genetic effects in multi-environment analysis. All models were fitted using Residual Maximum Likelihood (REML) (Patterson and Thompson 1971) using GenStat 13.3 (Payne et al. 2010).

Marker analysis and linkage map construction

A genetic map was constructed with 255 Diversity Arrays Technology (DArT) markers (Jaccoud et al. 2001), 83 SSRs, 5 sequence-tagged site (STS) and one RFLP marker. DArT markers were generated with a mini inverted repeat transposable element (MITE) and a *Pst*I library (Mace et al. 2009) and were genotyped according to Mace et al. (2008). Genomic DNA was isolated from approximately 500 mg of leaf tissue from each line using the protocol described by Saghai-Maroof et al. (1984). The RFLP marker, *isu52*, was genotyped as described in Magalhaes et al. (2004), while genotyping with the STS marker, *CTG29*, was as described in Caniato et al. (2007). The STS fluorescent markers *DG1*, *EM1*, *M1672* and *M9612* (sequences in Table S1) were amplified in multiplex and combined as *DG1/EM1* and *M9612/M1672* in reactions containing 30 ng of genomic DNA, 1X PCR buffer, 0.5 µM dNTP, 0.2 µM MgCl$_2$, 1.0 pmol of each 6FAM primer, 2.0 pmol of each HEX primer and 0.5 U of Taq polymerase (Phoneutria, Belo Horizonte, MG) in a final volume of 20 µL. Amplification for *DG1/EM1* consisted of an initial denaturation step at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min and a final extension step of 10 min at 72°C. Amplification for *M1672/M9612* consisted of 30 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 45 s. The amplification products were diluted and resolved in the automatic sequencer ABI3100 (Applied Biosystems, Foster City, CA).

PCR reactions for non-fluorescent SSRs followed the same protocols described above but using 2.5 pmol of each primer and 30 mM MgCl$_2$, with amplification products being resolved in 10% polyacrylamide gels with silver staining. The primer sequence for SSRs were reported in Brown et al. (1996), Bhattramakki et al. (2000), Kong et al. (2000) and in http://sat.cirad.fr/sat/sorghum_SSR_kit.

The physical position of SSR, STS and DArT markers were obtained by sequence similarity analysis using BLASTN (Altschul et al. 1997, with the filter option on and $E = 10$ in the case of primer sequences) against the sorghum genome (http://www.phytozome.net/sorghum). The

DArT sequences were kindly provided by Dr. Jean François Rami (Centre de Coopération Internationale en Recherche Agronomique Pour Le Développement—CIRAD). The resulting physical positions in addition to the consensus genetic positions for DArT markers (Mace et al. 2008, 2009) were used to validate the genetic map.

The genetic map was constructed using version 2.0-1 of the Onemap software (Margarido et al. 2007) that also includes options for multipoint analysis in RIL populations. In short, two-point analysis was used to obtain the maximum likelihood estimates of the recombination fractions between all pairs of markers. Next, markers were assigned to linkage groups using a LOD threshold of 5 and maximum distance of 30 cM with the Kosambi mapping function (Kosambi 1944). To order markers within chromosomes, functions "order" and "ripple" were used in order to obtain multi-point estimates of distances and likelihoods, in a similar way to MAPMAKER/EXP software. Prior physical and genetic information were used to consolidate marker orders and for assigning linkage groups to sorghum chromosomes as well as to discard poorly fitted markers. The nomenclature for chromosomes was based on Kim et al. (2005) and was the same used in the integrated map described in Feltus et al. (2006). Linkage groups split by gaps exceeding 30 cM were joined together when our previous genetic and physical information provided support for linkage group assignment to the same chromosome in a given orientation.

QTL analysis

A genome scan for the individual environments was performed using Simple Interval Mapping (SIM, Lander and Botstein 1989), extending the mixed model for the phenotypic analyses explained earlier. Our QTL mapping procedures were equivalent to regressing the random genetic effects, as defined above, on genetic predictors representing functions of conditional QTL genotype probabilities (Haley and Knott 1992; Jiang and Zeng 1997; Lynch and Walsh 1998; Boer et al. 2007). Evaluations for QTL presence along the genome were done with a maximum gap of 2 cM. In more detail, genotypic effects, $\underline{G}_i$, were partitioned into a QTL part and a residual: $\underline{G}_i = x_{iq}\alpha_q + \underline{g}_i$, with x_{iq} the genetic predictor and α_q the QTL effect and $\underline{g}_i$ denoting the residual genotypic effect after adjustment for the putative QTL effect. At marker positions, x_{iq} takes value $+1$ if the allele comes from SC283 and -1 otherwise, where q is the evaluation position of the putative QTL and i refers to the genotype ($i = 1\ldots90$). For testing QTL presence, Wald tests were used (Verbeke and Molenberghs 2000; Boer et al. 2007). The general QTL models were further extended to

accommodate the complete and incomplete block designs used in 2006 and 2007, respectively.

SIM was followed by composite interval mapping (CIM, Zeng 1994; Jansen and Stam 1994), where QTL detected by SIM were used as cofactors. We removed a cofactor from the model when the evaluation position for a QTL was within 15 cM of the cofactor.

Following up on the single environment mixed model QTL analyses, a multi-environment QTL analysis was performed to study QTL effects across environments (Boer et al. 2007). For that, the genotypic effects were partitioned in environment specific QTL effects and environment specific residual genetic effects: $\underline{G}_{il} = x_{iq}\alpha_{iql} + \underline{g}_{il}$, with α_{iql} the environment specific QTL effects at genomic position q and $\underline{g}_{il}$ an environment specific genetic residual. After scanning the genome using a procedure allowing for environment specific QTL effects (i.e., testing for QTL main effect and QEI together), it was tested for genomic positions with significant QTL whether there was indeed QEI present or just QTL main effects (consistent QTL expression across environments). Finally, to investigate possible confounding of QTL for phenology with those for grain yield and stay green QTL, as well as to study the association between plant height and grain yield QTL, flowering time and plant height QTL were used as cofactors in the multi-environment model described above.

A genome wide threshold of 0.01 was used to select QTL in both SIM and CIM for single and multi-environment analysis. The percentage of the genetic variance explained by, respectively, a single QTL and the full set of QTL was derived from the environment specific genetic variance in the multi-environment mixed model. Note, that this environment specific genetic variance represents a kind of residual genetic variance in a model that contains QTL. The variance explained by the full set of QTL was calculated as $100 \times [1 - \{(\text{genetic variance in model including the full set of QTL})/(\text{genetic variance in model without QTL})\}]$ and the percentage explained by a single QTL was calculated as $100 \times [(\text{genetic variance in model with full set of QTL except the one under evaluation}) - (\text{genetic variance in model with full set of QTL})/(\text{genetic variance in model without QTL})]$.

Results

The parental lines, BR007 and SC283, differed for grain yield (Fig. 1). Although the difference in stay green was small, SC283 has consistently shown higher stay green compared to BR007 in other experiments conducted across different years. The yield potential of BR007 was much greater than SC283 in control conditions. Although drought stress resulted in comparatively more similar grain yield for both lines, it was still higher in BR007. Thus, drought stress reduced grain yield in BR007 by ~50% whereas grain yield in SC283 remained 83% of that in well watered control. Stay green was similar for SC283 and BR007 but slightly higher for the former. Transgressive segregation was detected for both grain yield and stay green as observed in the 2007 trial under drought stress conditions (Fig. 2).

The two parents were similar in terms of both flowering time and plant height, resulting in progeny with limited segregation for both traits (Fig. S1). In addition, RILs showing strikingly different flowering time and plant height phenotypes were in general rare. The scatter plots shown in Fig. S2 show that yield and plant height were correlated as was the case for stay green and flowering time (maximum r of 0.49 and 0.34, respectively, both in WS06), suggesting confounding of phenology and plant height with grain yield and stay green.

The severity of drought stress to which the RILs were subjected was different between years, with 19 and 33% yield reduction in 2006 and 2007, respectively (Table 1). This response was probably caused by the occurrence of an occasional rain during the drought stress period in 2006, which did not happen in 2007. In addition, a smaller green leaf percentage was also observed in 2007, which agrees with a stronger drought stress in this year. Genetic variance estimates for grain yield were larger under control conditions in both years (Table 1). Heritability estimates for grain yield and stay green were intermediate to high, of approximately 0.67 for stay green in both years and ranging from 0.68 (WS06) to 0.82 (WW06) for grain yield.

Because the number of environments in this study was not excessive, we decided to adopt the unstructured model for the VCOV matrix to allow for maximum flexibility so that a specific genetic variance could be assigned to each environment and a specific covariance for each pair of environments. Genetic correlations for grain yield under control and water stress conditions in 2006 and 2007 and for stay green across years were consistently high, ranging from approximately 0.7 to 0.8 (Table 2).

QTL mapping was undertaken with a 344 loci genetic map including DArTs, SSRs, STSs and one RFLP, covering a total map distance of 2,033.7 cM across the ten sorghum chromosomes. QTL analysis was performed considering all environments simultaneously and the results are presented in Table 3 and Fig. 3. The grain yield QTL, Gy8, is probably related to differences in yield potential between the two parents as it was only expressed under control conditions in both years. Gy9, which was detected at one of the highest $-\log_{10}(P)$ values, explained a substantial proportion of the genetic variance in well watered and stress conditions in both years. This QTL showed a pronounced effect in grain yield, which averaged ~ 0.3 t ha^{-1}.

Fig. 1 Phenotypic means for grain yield (t ha^{-1}) and stay green (% green leaf area) for the parental lines, BR007 and SC283. Grain yield was assessed both in well watered (WW) and water stress (WS) conditions whereas stay green was only assessed under drought stress. Data was collected in 2007. *Vertical bars* represent the standard deviation
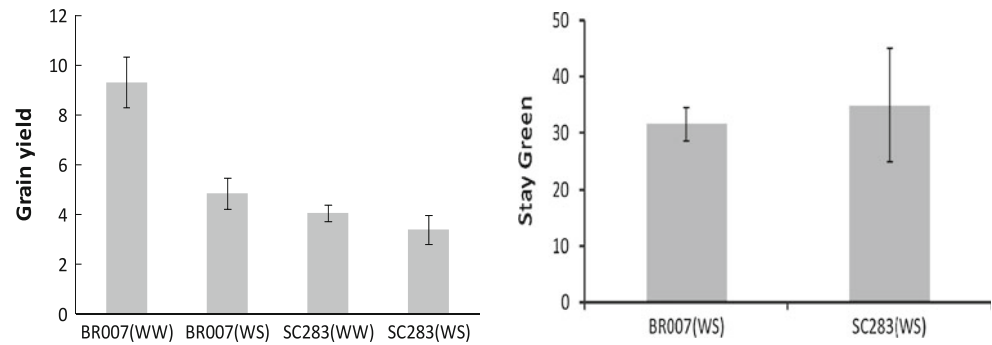
Fig. 2 Histograms showing the distribution of grain yield (t ha^{-1}) and stay green (% green leaf area) across 90 RILs subjected to drought stress in 2007. *Arrows* represent the phenotypic means for the parental lines based on three replications per parental line
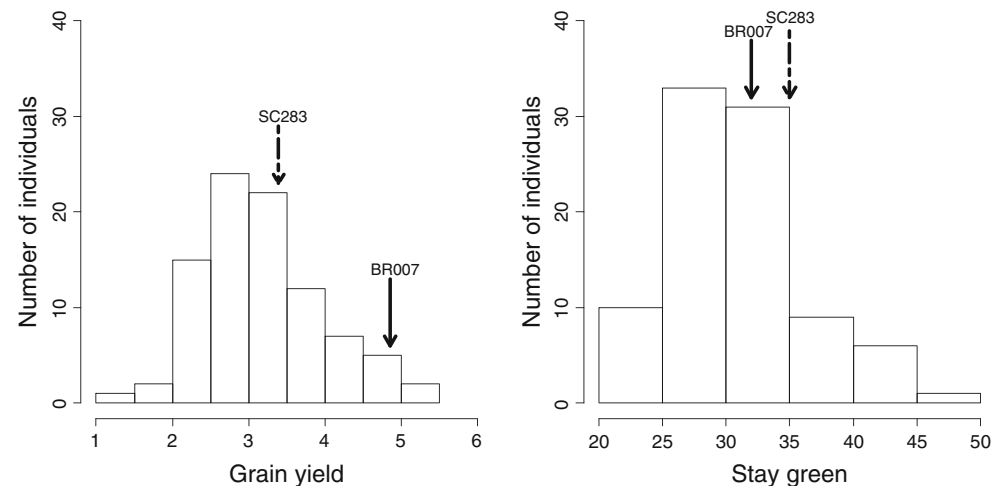
Table 1 Estimates of genetic (σ_g^2), replicate (σ_r^2), blocks within replicate ($\sigma_{r(b)}^2$) and error (σ_e^2) variances and their respective standard errors (between parenthesis)

Effects	Well water (WW)	Water stress (WS)	
	Grain yield	Grain yield	Stay green
	2006		
σ_g^2	1.50 (0.27)	0.66 (0.15)	66.90 (14.82)
σ_r^2	0.02 (0.03)	0.03 (0.04)	2.50 (0.98)
σ_e^2	0.95 (0.10)	0.89 (0.09)	59.18 (11.80)
Mean	5.61 (0.10)	4.56 (0.07)	54.73 (0.99)
h^2	0.82	0.68	0.68
	2007		
σ_g^2	1.24 (0.25)	0.45 (0.10)	20.00 (4.57)
σ_r^2	0.0	0.07 (0.08)	0.0
$\sigma_{r(b)}^2$	0.01 (0.05)	0.02 (0.03)	3.57 (2.09)
σ_e^2	1.14 (0.13)	0.57 (0.06)	26.79 (3.08)
Mean	4.86 (0.10)	3.24 (0.07)	32.35 (0.43)
h^2	0.76	0.70	0.67

Phenotypic means and heritability (h^2) estimates are shown for grain yield (t ha^{-1}) and stay green (% green leaf area) in well watered (WW) and water stress (WS) conditions in 2006 and 2007

Although strict drought tolerance QTL, that is, QTL expressed only under drought stress conditions were not detected, *Gy6-1* explained a large portion of the genetic variance specifically under water stress in 2006 ($\sim$18%) and 2007 ($\sim$10%) whereas only a neglectable fraction was explained in non-stress environments. Similarly, *Gy9* was also substantially responsible for the genetic variance under drought stress. However, this QTL appeared to be less specific to drought stress than *Gy6-1* as nearly 10% of the genetic variance was explained by *Gy9* also under control conditions in both years.

In general, multi-environment analysis uncovered both environment specific and main effect QTL and the majority of the alleles increasing grain yield were donated by the parent, BR007, consistent with its higher yield potential compared to SC283 (Fig. 1). One interesting exception was the main effect QTL located at position 2 cM on chromosome 3 (*Gy3-1*). At this locus, the SC283 allele increased grain yield by $\sim$0.2 t ha^{-1} and *Gy3-1* explained $\sim$7 and $\sim$10% of the genetic variance under drought stress in 2006 and 2007, respectively.

Eight QTL associated with stay green were mapped to chromosomes 2 (two QTL), 3, 4, 5, 6, 8 and 9 (Table 3;

Table 2 Genetic correlations for grain yield (t ha^{-1}) and stay green (% green leaf area) between environments using multi-environment analysis and the unstructured model to estimate the genetic variance–covariance matrix

Environment	Grain yield			Stay green
	WW06	WS06	WW07	WS06
WS06	0.82			
WW07	0.72	0.81		
WS07	0.69	0.80	0.76	0.80

WW06 well watered 2006, *WS06* water stress 2006, *WW07* well watered 2007, *WS07* water stress 2007

Table 3 Estimates of QTL effects using multi-environment QTL analysis for grain yield (t ha^{-1}) and stay green (% green leaf area) without flowering time/plant height cofactors

QTL	QTL position (cM)	Closest markers	Multi-environment								*F*	Avse
			WW06		WS06		WW07		WS07			
			Effects	%GV	Effects	%GV	Effects	%GV	Effects	%GV		
			Grain yield									
Gy2	194.0	sPb3361–Xtxp348	−0.272[a]	8.57	−0.272	3.69	−0.272	2.30	−0.272	8.86	4.68	(0.08)
Gy3-1	2.0	sPb0965–Xcup61	0.215[a]	<1.00	0.215	7.21	0.215	4.10	0.215	10.34	5.43	(0.07)
Gy3-2	102.2	sPb0357–M340711	−0.173	<1.00	–	–	0.139	<1.00	−0.107	2.34	4.88	(0.10)
Gy4	186.0	sPb6098–sPb0110	−0.184	1.40	–	–	−0.261	3.52	−0.120	2.20	4.25	(0.11)
Gy6-1	28.0	sPb1635–sPb8060	−0.343[a]	<1.00	−0.343	17.58	−0.343	<1.00	−0.343	9.53	5.17	(0.09)
Gy6-2	65.5	Xtxp145–sPb8928	−0.162	1.08	0.147	1.40	–	–	−0.143	3.90	5.18	(0.10)
Gy8	112.0	Xtxp321–sPb4546	−0.349	4.84	–	–	−0.376	7.18	–	–	7.44	(0.12)
Gy9	218.0	M343363–sPb4087	−0.308[a]	10.33	−0.308	24.14	−0.308	10.45	−0.308	9.80	6.10	(0.07)
Gy10	4.0	Xcup49–Xcup42	−0.578	14.46	–	–	–	–	−0.174	3.88	3.78	(0.12)
		Total (%)		**37.42**		**42.24**		**28.75**		**60.93**	4.68	
			Stay green									
St2-1	109.0	sPb2685–sPb1801			1.096[a]	4.08			1.096	4.90	5.12	(0.46)
St2-2	226.3	M188941–M188566			−0.880	<1.00			1.271	5.91	5.27	(0.75)
St3	18.0	ISEP0107–sPb2839			4.771	29.74			–	–	10.36	(0.75)
St4	112.0	sPb8806–M340487			3.603	17.05			1. 472	8.05	10.23	(0.74)
St5	98.3	sPb1989–sPb6258			−4.383	26.28			–	–	7.00	(0.73)
St6	30.0	sPb1635–sPb8060			1.045[a]	<1.00			1.045	2.25	7.32	(0.58)
St8	98.0	sPb1661–Xtxp321			2.344[a]	<1.00			2.344	14.46	15.93	(0.59)
St9	218.0	M343363–sPb4087			2.547[a]	3.49			2.547	22.79	11.69	(0.52)
		Total (%)				**69.25**				**65.15**		

QTL are coded as grain yield (*Gy*) or stay green (*St*) and multiple QTL within the same chromosome were numbered sequentially according to the chromosomal positions. Average standard errors (Avse) for each effect are shown between parentheses. Main effects are shown for QTL whose QEI was not significant at $P < 0.05$. %GV stands for the percentage of the genetic variance that is explained by a given QTL (the percentage of the genetic variance explained after fitting all significant QTL in the model is shown in bold, following individual %GV estimates). (–) QTL with effects below the Avse or above the Avse by up to 0.03 and explaining less than 1% GV were considered of questionable support and were thus presented separately in Table S2

WW06 well watered 2006, *WS06* water stress 2006, *WW07* well watered 2007, *WS07* water stress 2007

[a] QTL main effects

Fig. 3). Differently than in the case of grain yield, the majority of the superior alleles for stay green were derived from SC283, except for *St2-2* and *St5* both in WS06. Once again for stay green, QEI was a common event. The three QTL explaining the largest proportion of the phenotypic variance had alleles increasing stay green coming from SC283 (~30% for *St3* in 2006 and *St9* in 2007 with ~23%) and BR007 (*St5* in 2006 with 26%). The proportion of the genetic variance explained by the final multi-QTL model in each environment for grain yield varied between ~30% (WW07) and 60% (WS07) and from 65% (WS07) to 70% (WS06) for stay green (Table 3).
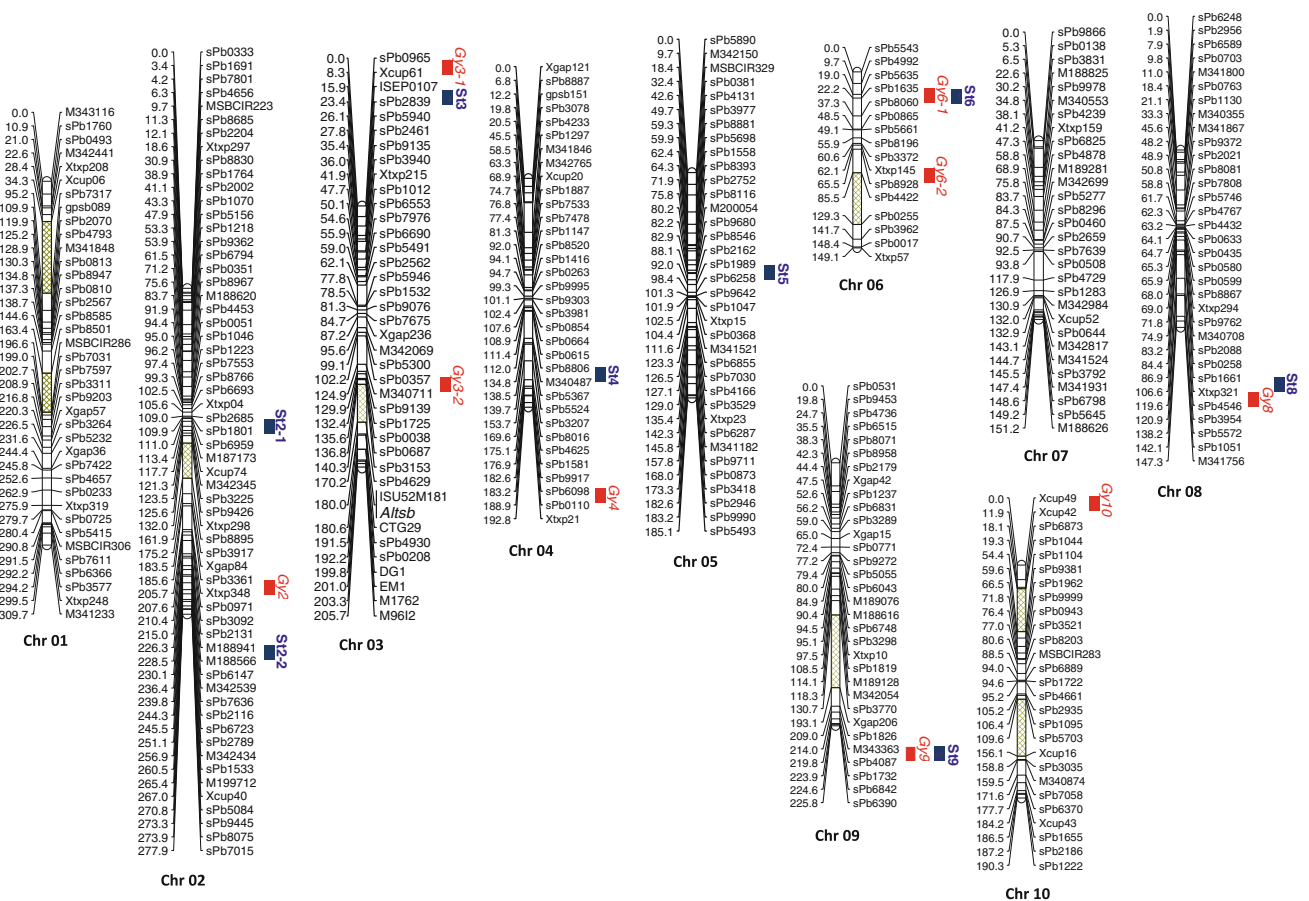
Fig. 3 Sorghum linkage map developed using the RIL population of the cross BR007xSC283. The positions for QTL influencing grain yield (*Gy*, in *blue*) and stay green (*St*, in *red*) as estimated by multi-environment analysis without flowering time/plant height cofactors are shown. Chromosome (Chr) designations were assigned according to the integrated map described by Feltus et al. (2006). Cross-hatched squares depict regions with genetic distances exceeding 30 cM. The markers beginning with *M* and *sPb* are DArT markers developed from MITE and *Pst*I libraries, respectively (color figure online)

We subsequently undertook QTL analysis for both flowering time and plant height in the BR007 × SC283 RIL population and the results are shown in Fig. S3. All flowering time QTL were detected in the vicinity of previously detected grain yield QTL at varying linkage intensities and three were near stay green QTL. Those are located at positions 236 cM on chromosome 2 (at 42 cM from *Gy2* and 9.7 cM from *St2-2*), 100 cM on chromosome 3 (at 2.2 cM from *Gy3.2*), 150 cM on chromosome 4 (36 cM from *Gy4* and 38 cM from *St4*) and at position 204 on chromosome 9, 14 cM from both Gy9 and St9. Four QTL were detected for plant height but in general at lower $-\log_{10}(P)$ values compared with flowering time QTL except for the plant height QTL at position 216 cM on chromosome 9. Thus, a cluster of four rather tightly co-localized QTL was comprised by the plant height QTL a position 216 cM, the grain yield QTL, *Gy9*, at position 218 cM, the flowering time QTL at position 204 cM and also the stay green QTL, *St9* at 218 cM. In addition, the plant height QTL on chromosome 6 was found within the 28–65.5 cM interval delimited by *Gy6-1* and *Gy6-2*.

Differences in phenology, mostly variations in flowering time, may affect the expression of traits related to drought tolerance such as grain yield and stay green in sorghum. Here we detected general co-localization between all flowering time and four grain yield in addition to three stay green QTL, including a QTL controlling both flowering time and plant height on chromosome 9. In addition, the plant height QTL on chromosome 6 was located near two grain yield QTL. These results prompted us to more formally investigate the possibility that variations in phenology were underlying some of the grain yield and stay green QTL and a possible association between QTL for plant height and grain yield. To this purpose we conducted multi-environment QTL mapping with a model including the flowering time and plant height QTL as cofactors. The QTL profiles for grain yield and stay green are shown in Figs. 4 and 5, respectively, without and with flowering time/plant height cofactors, and QTL effects with cofactors are shown in Table 4. The QTL *Gy3-2*, *Gy4*, *Gy6-2* and *Gy9* were not detected in the model including flowering

cofactors (Fig. 4), suggesting these grain yield QTL to be related to variations in flowering time and plant height. However, *Gy2*, *Gy3-1* at position 2 cM, *Gy6-1*, *Gy8* and *Gy10* cannot be explained by variations in flowering time/plant height and may thus be related to mechanisms underlying yield potential and/or drought tolerance (Fig. 4). The same rationale indicates that the stay green QTL *St2-1*, *St2-2*, *St5*, *St6* and *St9* can be attributed to variations in flowering time/plant height whereas the remaining stay green QTL may underline mechanisms responsible for green leaf maintenance under water limitation after flowering (Fig. 5). When the variation attributed to the detected flowering time and plant height QTL was removed from the model, the grain yield QTL, *Gy3-1* and *Gy6-1*, in addition to *St3* and *St8* stood out under water stress conditions, explaining a large proportion of the total genetic variance for the respective traits (Table 4). Two cases of co-localization between stay green and grain yield QTL were detected for *Gy3-1* and *St3* (2–26 cM) and *Gy8* and *St8* (110–102 cM) although both the effect and variance explained by *Gy8* in water stress environments was extremely low. Only in the case of the QTL on chromosome 3, which showed pronounced expression in water stress conditions, did the allele increasing phenotypic expression consistently come from the same parent, SC283 in this case.

Discussion

The parents of the RIL population were the inbred lines, BR007 and SC283, which are both non-restorer B lines in cytoplasm A1. Both parents and derived RILs are 3-dwarf types and the parents show similar flowering time and plant height. SC283 shows aluminum tolerance, excellent resistance to foliar diseases such as leaf rust and anthracnose, and has vitreous endosperm with improved weathering resistance. The second parent, BR007, is a high yielding elite breeding line in the Embrapa Maize and Sorghum program, and was selected across a wide array of marginal stress environments, thus showing very good adaptation to the Brazilian conditions. The identification of QTL related to performance under drought stress was thus purposely undertaken in a RIL population that is highly relevant for breeding purposes as an attempt to narrow down the gap between QTL identification and their eventual utilization in breeding drought tolerant sorghums. BR007 presented high yield in well watered conditions but a strong reduction occurred after the imposition of drought stress. Grain yield in SC283 was lower than BR007 both in control and water stress conditions but this line presented yield stability, with very little yield reduction caused by drought stress. It should be noted that the stress intensity in this study was
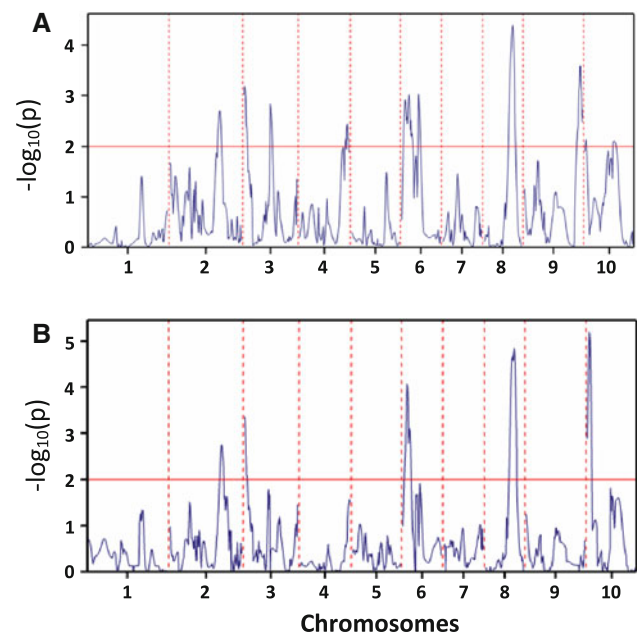

Fig. 4 Graphical display of the QTL detected by multi-environment analysis for grain yield without (**a**) and with flowering time/plant height cofactors (**b**). The associated tail probability of the Wald statistics, *P*, is expressed as $-\log_{10}(P)$, analogous to the usual LOD score profile. The *red horizontal line* is the 1% threshold (color figure online)
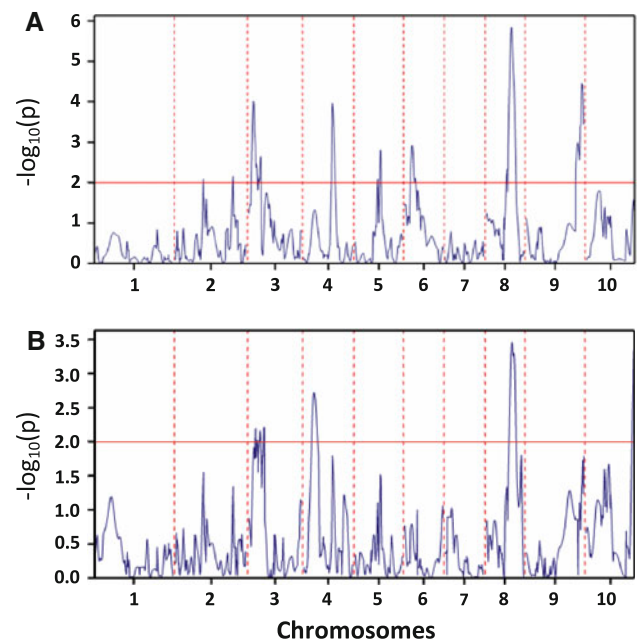

Fig. 5 Graphical display of the QTL detected by multi-environment analysis for stay green without (**a**) and with (**b**) flowering time/plant height cofactors. The associated tail probability of the Wald statistics, *P*, is expressed as $-\log_{10}(P)$, analogous to the usual LOD score profile. The *red horizontal line* is the 1% threshold (color figure online)

clearly agronomically relevant, resulting in a yield reduction of approximately 1 and 1.6 t ha^{-1} in 2006 and 2007, respectively. Our experiments were conducted within a

Table 4 Estimates of QTL effects using multi-environment QTL analysis for grain yield (t ha^{-1}) and stay green (% green leaf area) with flowering time and plant height cofactors

QTL	QTL position (cM)	Closest markers	Multi-environment								F	Avse
			WW06		WS06		WW07		WS07			
			Effects	%GV	Effects	%GV	Effects	%GV	Effects	%GV		
			Grain yield									
Gy2	200.0	sPb3361–Xtxp348	−0.190[a]	4.95	−0.190	<1.00	−0.190	1.90	−0.190	4.32	4.01	(0.09)
Gy3-1	2.0	sPb0965–Xcup61	–	–	0.278	10.02	0.317	5.61	0.234	12.02	4.29	(0.12)
Gy6-1	16.0	sPb4992–sPb5635	−0.381	4.76	−0.456	21.05	−0.126	<1.00	−0.185	4.91	4.93	(0.14)
Gy8	110.0	Xtxp321–sPb4546	−0.458	10.36	–	–	−0.363	6.80	–	–	5.06	(0.12)
Gy10	8.0	Xcup49–Xcup42	−0.645	16.41	−0.152	1.19	–	–	−0.168	3.16	5.86	(0.14)
		Total (%)		**40.30**		**34.91**		**14.39**		**27.56**		
			Stay green									
St3	26.0	sPb2839–sPb5940			4.293	29.90			1.041	5.31	7.02	(0.97)
St4	40.0	sPb4233–sPb1297			3.317	10.79			–	–	6.67	(1.18)
St8	102.0	sPb1661–Xtxp321			–	–			2.121	19.70	10.51	(1.08)
St10	187.2	sPb1655–sPb2186			−2.003	5.41			1.260	10.12	10.80	(0.90)
		Total (%)				**35.15**				**30.76**		

QTL are coded as grain yield (*Gy*) or stay green (*St*) and multiple QTL within the same chromosome were numbered sequentially according to the chromosomal positions. Average standard errors (Avse) for each effect are shown between parentheses. Main effects are shown for QTL whose QEI was not significant at $P < 0.05$. %GV stands for the percentage of the genetic variance that is explained by a given QTL (the percentage of the genetic variance explained after fitting all significant QTL in the model is shown in bold, following individual %GV estimates). (–) QTL with effects below the Avse or above the Avse by up to 0.03 and explaining less than 1% GV were considered of questionable support and were thus presented separately in Table S3

WW06 well watered 2006, *WS06* water stress 2006, *WW07* well watered 2007, *WS07* water stress 2007

[a] QTL main effects

location that is representative of the Brazilian semi-arid region. While it is possible that our findings prove applicable to other regions with similar environmental conditions, further validation in the target environment is a necessary step in order to conscientiously decide about potential breeding applications in other areas.

One way to determine the prospects for gains in breeding programs relates to the proportion of the phenotypic variance that is explained by the genetic component, which can be assessed by the heritability coefficient. Heritability estimates in this study were high in control conditions and only slightly lower under drought stress which in our case can be explained by a decrease in genetic variance under drought stress. High heritability estimates were also found in other studies for grain yield in well watered conditions (Srinivas et al. 2009) and stay-green in water stress conditions (Xu et al. 2000; Kebede et al. 2001).

Multi-environment analysis revealed nine and eight QTL for grain yield and stay green, respectively, and provided a means to formally test for QEI. The occurrence of environment-specific QTL was clearly rather the rule than the exception in this study as strong evidence for QEI was the case for nine out of the seventeen QTL identified considering both traits. This is by no means surprising

considering the high environment interaction typically associated with the expression of drought tolerance (Tuinstra et al. 1996), highlighting the importance of multi-environment analysis for mapping QTL underlying drought tolerance in sorghum. As noted by Korol et al. (1998), ignoring possible variation of QTL effects among environments may lead to erroneous decisions in latter applications of the QTL mapping results. Ignoring genetic correlation can lead to overoptimistic inferences such as spurious QTL detection and inappropriate standard errors for parameter estimates (Piepho 2000).

The grain yield QTL, *Gy8*, was found to be related to intrinsic differences in yield potential between the two parents, as it was only detected in control conditions. The majority of the alleles increasing grain yield were derived from BR007, consistent with the high yield potential of this elite line. QTL conserved across years such as *Gy8*, may eventually be considered as a target for molecular breeding strategies only in environments with low probability of drought stress. Interestingly, *Gy3-2* showed a crossover-type QEI, with alleles increasing grain yield coming from BR007 in 2006 and SC283 in 2007. Another crossover interaction was found for *Gy6-2* that is located in the same region of chromosome 6 as a grain yield QTL identified by

Srinivas et al. (2009), since they are both flanked by the same SSR marker, *Xtxp145*, and for the stay green QTL, *St2-2*. Selection for a QTL under strong GEI should not be a priority unless it can be shown that they are particularly important in the target environment for the crop (Clarke et al. 1992) and provided the environmental conditions leading to the crossover response can be pinpointed. Significant crossover interactions were reported for six yield QTL in maize by Boer et al. (2007). These authors indicated that most of the observed QEI effects could be explained by differential QTL expression dependent on longitude or year, with temperature being the underlying basis for these responses.

According to Quinby (1974), plant height in sorghum is governed by four, non-linked, brachytic dwarfing genes, *Dw1–Dw4* with tallness being partially dominant to shortness. Accordingly, tall or zero-dwarf types may be three to four meters in height whereas 4-dwarf plants may grow only to be one meter. *Dw3*, which is common in commercial sorghum lines, has been cloned and is homologous to maize *BRACHYTIC 2* (*Br2*), which encodes a protein similar to adenosine triphosphate (ATP)-binding cassette transporters of the multidrug resistant (MDR) class of *P*-glycoproteins (Multani et al. 2003). The causative loss of function mutation in *dw3* is likely an 882 bp direct duplication in exon 5, probably resulting in impaired polar auxin transport analogous to that observed for *br2* in maize. More recently, Brown et al. (2008) conducted association analysis in sorghum and found that a second locus, *Sb-HT9.1*, which had been previously reported to affect plant height in different studies, is epistatic to *Dw3*. The most significant association was found with an SSR marker at position 57.21 Mb on chromosome 9. Interestingly, the plant height QTL detected on chromosome 9 in the present study is located in the 176–216 cM interval, within which the SSR marker, *Xgap206*, lies at position 193.1 cM. Sequence similarity analysis with the sorghum genome indicated that *Xgap206* is located at the physical position 59.16 Mb, thus only 1.95 Mb away from the marker yielding maximum association with plant height traits in Brown et al. (2008). Considering that even the farthest markers that were tested at positions near 59 Mb showed significant *P* values in the Brown et al. study, *Sb-HT9.1* and the plant height QTL detected in the present study are possibly the same. If so, here we show that *Sb-HT9.1* also controls variation in flowering time in addition to plant height given the close co-localization to QTL controlling those two traits in the present study. Two overlapping QTL affecting both plant height and flowering time located in the same general position as *Sb-HT9.1* were also reported by Lin et al. (1995) on sorghum chromosome 9. The grain yield QTL, *Gy9*, and *St9*, underlying stay green variation, were also tightly co-localized in the *Sb-HT9.1* region in our

model without flowering time/plant height cofactors. Therefore, considering that removing the variation that is likely due to *Sb-HT9.1* in the QTL model for stay green and grain yield led to the loss of the previously detected QTL for both traits (*Gy9* and *St9*), here we illustrate the importance of controlling for differences in phenology and plant height as they may be an important source of false positives in QTL mapping targeting drought tolerance.

Another interesting result pertaining to a possible confounding effect of phenology-related QTL was found for stay green. The QTL *St2-2* was detected at position ∼226 cM on chromosome 2, near the DArT marker *SPb-2131* at 215 cM. According to Mace and Jordan (2010), the maturity gene, *Ma5*, is closely linked to the SSR markers *txp429* and *txp431*. We were able to project the primer sequences for both SSR loci on the sorghum genome and found they delimit a physical region between positions 68.41 Mb (*txp431*) and 68.85 Mb (*txp429*), thus very close to the physical position 68.3 Mb where *SPb-2131* lies on chromosome 2. Upon the addition of flowering time QTL as cofactors including a QTL at position 236 cM, thus very close to *St2-2*, this stay green QTL was no longer detected. This strongly suggests that flowering time variation encoded by the *Ma5* locus was in fact responsible for *St2-2*.

Due to transgressive segregation, variations in phenology are expected even if the parents of the mapping population do not show differences for these traits. Staggered planting of the population based on groups that are more homogeneous in phenology, so that the onset of drought stress is uniform in terms of flowering stage is an option but is also operationally difficult. Therefore, a statistical approach based on the adoption of proper phenology-related cofactors in the QTL models appears to be an efficient strategy to help controlling for false positives in drought tolerant studies.

Another dwarf locus, *Dw2*, is linked to the maturity locus, *Ma1*, within a 13.5–29.2 cM interval on sorghum chromosome 6 (Klein et al. 2008) or 14.2–21.2 cM according to the map available at http://sorgblast3.tamu.edu/pklein.htm. From this last resource, the marker *Xtxp434* is located at the position 16.2–19.7 cM, thus within the *Dw2-Ma1* region, and the physical position for this marker is 42.61 Mb. The plant height QTL detected on chromosome 6 is located within a 42–52 cM window in our map and sequence similarity analysis allowed us to obtain the physical position for the DArT marker, *sPb8060*, at 45.46 Mb on chromosome 6 (37.3 cM), placing it only 2.85 Mb away from *Xtxp434*. This suggests that the plant height QTL detected on chromosome 6 in the present study and *Dw2* might be the same, and that *Ma1* was probably monomorphic between the RIL parents as no flowering time QTL was detected on chromosome 6. Our multi-environment QTL model detected the grain yield QTL,

Gy6-1, only 2 cM from *St6* that controls stay green. However, using flowering time/plant height cofactors, *Gy6-1* remained being detected at a similarly high $-\log_{10}(P)$ value but the probability for *St6* no longer exceed our $P < 0.01$ cutoff. Along with the fact that *Gy6-1* had a pronounced effect in grain yield particularly under drought stress in 2006, this suggests that the potential mechanism underlying this QTL is not related to the maintenance of green leaves under drought. This is reinforced by the fact that in the absence of flowering time/plant height cofactors the alleles increasing phenotypic expression came from alternate parents, BR007 for *Gy6-1* and SC283 for *St6*, which would imply a detrimental effect of stay green in grain yield under drought conditions. Alternatively, grain yield advantage at this locus may arise as a trade-off between biomass production and tallness as proposed for 'green revolution' genes (Peng et al. 1999), with *dw2*-bearing plants showing improved grain yield under drought conditions.

Another interesting outcome of including flowering time/plant height cofactors in QTL mapping procedures relates to the detection of crossover QEI. Without these cofactors, three QTL showing crossover-type QEI were detected, namely *Gy3-2*, *Gy6-2* and *St2-2*. However, upon the addition of flowering time/plant height cofactors, these three QTL were no longer detected. Variations in temperature have been recognized as a critical factor underlying QEI (Malosetti et al. 2004; Boer et al. 2007) and minimum temperature during flowering was the environmental covariable that explained nearly 80% of the QEI for grain yield in maize (Vargas et al. 2006). Given the known relationship between temperature variations and phenology, this suggests that variations in flowering time may be the most important factor leading to crossover-type interactions in the present study.

For stay green, *St5*, which was not detected with flowering time/plant height cofactors in the present study is likely the same as *Stg4* located on chromosome 5, near *Xtxp15* (Harris et al. 2007). This QTL was also reported by Xu et al. (2000), Subudhi et al. (2000), Kebede et al. (2001) and Srinivas et al. (2009). Evidence for grain yield advantage coming from stay green was found on chromosome 3, as *Gy3-1* was co-localized with *St3*, with those two QTL being separated by 24 cM. Clearly, we cannot rule out the possibility for two linked QTL as opposed to a single pleiotropic QTL underlying both traits. However, most of the QTL for grain yield in this study had alleles increasing phenotypic expression coming from the high yielding parent, BR007, and not from SC283. Strikingly, one exception was exactly *Gy3-1*, where the positive allele was donated by SC283. Considering that the allele increasing stay green at *St3* also came from SC283, our current data suggest that *Gy3-1* and *St3* may be a single

pleiotropic QTL. Therefore, with this apparently novel stay green QTL, here we present evidence suggesting that breeding for stay green may result in yield advantage for sorghum cultivated under drought stress.

In the present study, the identification of molecular markers linked to QTL for stay green and grain yield is a starting point for the future development of molecular breeding strategies aimed at helping plant breeders to manipulate and pyramid QTL to improve drought tolerance in sorghum. A detailed characterization of these genomic regions through the development and evaluation of near-isogenic lines is expected to lead to a better understanding of drought tolerance in sorghum. In addition, in conjunction with targeted mapping in different populations and with additional environments, we expect to more precisely define the effect of the QTL detected here so that clear targets for marker-assisted selection to improve drought tolerance in sorghum can be defined.

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