

# QTL mapping and loci dissection for leaf epicuticular wax load and canopy temperature depression and their association with QTL for staygreen in *Sorghum bicolor* under stress

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**Abstract** Plant waxes and staygreen are distinct phenotypic traits that have been independently implicated in heat and drought tolerance among grasses. The association between these two traits has not been fully explored, which makes the exploitation of synergy between them difficult. This study assessed the association between QTL regulating the staygreen (Stg) trait in sorghum and those regulating epicuticular wax load (WL) and associated canopy temperature depression (TD). Using a recombinant inbred line (RIL) population derived from Tx642 and Tx7000, phenotypic data were collected in three replicated field trials and one greenhouse trial. High absolute TD generally corresponded to high WL. The  $r^2$  of TD against WL was highest under non-stress conditions in the greenhouse while it was much larger in the cooler

and irrigated field conditions compared to hotter, drier field trials. The genetic correlations between the two traits also followed this pattern. Composite interval mapping identified a total of 28 QTL, 15 of which had significant overlaps between different traits. Most of the wax QTL were associated with pre-anthesis drought tolerant Tx7000. However, one QTL for WL overlapped with a QTL for staygreen (*Stg2*) and was represented by a single, isolated marker near the centromeric region on the short arm of SBI-01. The marker is identified by a *Cis*-acting regulatory module and is part of a 2-kb multifunctional motif-rich region which includes core promoter and enhancer regions and transcription elements, including a drought-responsive MYB binding site. We suggest that this QTL may be pleiotropic for important stress tolerance mechanisms regulating both staygreen and leaf wax in sorghum.

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## Introduction

Drought is a major limiting factor in crop productivity. The capability of a plant leaf to sustain a healthy internal temperature in the face of erratic external

conditions is vital in maintaining leaf cell water balance, and necessary for optimal flowering, seed development, and biomass accumulation (McLaughlin and Boyer 2004; Kumari et al. 2007). Adequate water availability and slowed post-anthesis senescence (staygreen) have been shown to have a direct positive impact on yield, while leaf epicuticular wax has been shown to deter excessive water loss through the leaf cuticle under elevated air temperatures and water deficit (Clarke and Richards 1988). Investigating the genetic association between staygreen and plant surface waxes might shed light into the possible interaction of the two traits in alleviating the impacts of stress on crops.

In cereal crops like grain sorghum, the staygreen trait has traditionally been defined as the ability of a plant to maintain active photosynthetic processes under water deficit, especially post anthesis (Casta et al. 1999). When defined in terms of green leaf area, staygreen has been scored as a leaf death count (Xu et al. 2000) and more recently, leaf area under decline (LAUD) (Kumari et al. 2007), compared to a reference genotype (Thomas and Howarth 2000). This corresponds to the ability of a plant to maintain active photosynthetic processes with delayed senescence post-anthesis. In sorghum, staygreen has been associated with QTL that are stable across environments (Tao et al. 2000; Haussmann et al. 2002; Kassahun et al. 2010). In general, four major QTL for staygreen individually exhibit different tolerance to drought-induced senescence (Harris et al. 2007; Kassahun et al. 2010; Xu et al. 2000; Rosenthal and Gerek 1991), as defined by the ability of some sorghum genotypes to stay green during post-anthesis water stress (Blum 2005; Tao et al. 2000).

The induction of senescence involves a complex regulatory network in leaf senescence processes (He et al. 2002; Lim and Nam 2005; Lim et al. 2003; Noh and Amasino 1999; Quirino et al. 2000). Some senescence up-regulated transcription factor genes belong to various transcription factor families including SARK (senescence-associated receptor kinase), GTP-binding protein, WRKY (WRKY amino acid signature-containing DNA-binding domain), EREBP (ethylene responsive elements binding protein), NAC (no apical meristem), and MYB (myeloblastoma) families (Quirino et al. 2000; Yoshida et al. 2002). Of these, the genes for WRKY53, a MYB protein, and zinc finger protein show transiently increased

expression at a very early stage of leaf senescence (Buchanan-Wollaston et al. 2003; Guterman et al. 2003; Hinderhofer and Zentgraf 2001).

Because wax modulates canopy temperature and buffers against abiotic stress-induced injury and signal transduction, it may be important in maintaining the functionality of staygreen. Waxes thus may reduce stress-related stimuli for catabolic processes, thereby slowing natural aging and degradation of chlorophyll at the height of reproductive activity. The onset of wax accumulation is well known to be at early vegetative phases of many grass species (Clarke and Richards 1988). The synchrony of its peaking during florescence and anthesis in some plant species suggests a possible association with constitutive genetic control of staygreen and improved tolerance to heat and drought among tolerant variants. In this study, we have begun to test this relationship and the genetic association between the two traits.

## Materials and methods

### Germplasm, mapping population and evaluation environments

One hundred F<sub>12</sub> recombinant inbred lines (RILs) developed from a cross of Tx642 and Tx7000 were used in this study. Tx642 (B35) has been a useful source of staygreen for research and development of drought tolerant hybrids (Harris et al. 2007; Tao et al. 2000). It is an inbred BC<sub>1</sub> derivative line of IS12555 durra sorghum from Ethiopia, and varying dominance is exhibited by its genes regulating the staygreen trait (Xu et al. 2000; Sanchez et al. 2002). Tx642 is susceptible to pre-flowering drought stress but highly tolerant to post-flowering drought stress (staygreen trait). Tx7000 is an elite line released by the Texas Agricultural Experiment Station at Lubbock. It was derived from a 'Kafir' × 'Milo' cross, and was originally grown as a cultivar and then later used as a male parent in the late 1950s (Xu et al. 2000). It is currently a public line used in breeding programs in the United States (Subudhi et al. 2000). Tx7000 is pre-flowering drought tolerant and post-flowering drought susceptible (Xu et al. 2000).

A total of four environments were used to phenotype epicuticular wax load (WL) and canopy temperature depression (TD). The environments consisted of

three field trials at Texas A&M AgriLife Research Centers in College Station (irrigated), Weslaco (irrigated) and Corpus Christi (rainfed) in 2011. A fourth evaluation environment was in the greenhouse at College Station with normal watering in 2011. Each RIL was sown as three replicates in each field environment, and three replicates (pots) with two plants per pot in the greenhouse.

The field trials were sown in the spring of 2011: on February 16th for Weslaco, March 9th for Corpus Christi and on March 23rd for College Station. All plots were sown in randomized complete block design (RCBD) with each experimental unit composed of two contiguous rows 5.3 m in length with ~110–130 plants per row. The spacing between rows was 76 cm in Weslaco and College Station, and 1.0 m in Corpus Christi. Open furrow irrigation was applied in Weslaco and College Station over the growth period, while Corpus Christi was rain fed 1 day after sowing. All other standard agronomic practices were conducted as typical for the region. Canopy temperature readings and leaf samples for wax analysis were taken at 50% flowering, observed independently for each plot.

In the greenhouse, 100 RILs were sown in 30 cm diameter pots filled with potting mix (MetroMix 900TM forest peat moss, Schulenburg, Texas), on December 4, 2011. The pots were watered one d after sowing and at subsequent intervals of 4–6 days thereafter until flowering. No water was applied for the 2 days prior to each sampling date. Canopy temperature readings and leaf samples for wax analysis were taken on the same day at 50% anthesis on a plot basis. The mean air temperatures in the growth environments were taken to be the means averaged ~13 cm above the canopies on a plot basis (or pot basis in the greenhouse) between 12:00 and 14:00 using an external air temperature probe component of a digital infrared thermometer (IRT, see next section). The temperatures during the reproductive phase through the sampling period were as follows: greenhouse, late February 2011: ~24–26.5 °C; Weslaco, early June 2011: ~25–30 °C; Corpus Christi, late June 2011: ~39–41 °C, and College Station, mid July 2011: ~42–46 °C.

#### Phenotyping and design

Days to flowering were recorded for each plot. At 50% anthesis on a plot basis in the field environments, four

plants per replicate were randomly sampled and 10 fresh leaf disks, 7.0 mm in diameter, were punched from the flag leaf of each selected plant. The leaf disks were punched directly into clean vials, one vial per plant, using a handheld Precision Leaf Sampler, No. 11325 Series (IL, USA, [www.rabbittool.com](http://www.rabbittool.com)). All plants grown under greenhouse conditions were similarly sampled. A colorimetric method (Ebercon et al. 1977) was used to extract leaf epicuticular wax and the spectrometric absorbance was obtained at 290 nm using a PHERAStar FS spectrometer integrated with MARS Data Analysis Software V 3.10R3 (BMG LABTECH, Ortenberg, Germany; <http://www.bmglabtech.com>). Data were collected in late February 2011 (greenhouse), early June 2011 (Weslaco), late June 2011 (Corpus Christi) and mid July 2011 (College Station). The Beer-Lambert conversion algorithm (Paynter 1981) was used from a new standard curve to convert absorbance readings to wax load in  $\text{mg dm}^{-2}$ .

The timing for temperature sampling was during clear skies between 12:00 and 14:00 when the correlation of canopy temperature at anthesis and yield is highest (Balota et al. 2007a, b) and plant water stress is expected to be high (Balota et al. 2007a, b; Fan et al. 2005) for the stress-treated field environments. The leaf temperatures ( $T_l$ ) for all plants were averaged using a hand-held IRT (model OAKT on Pro, Class 2(ii) laser product, output wavelength 630–670 nm, with external probe for ambient air temperature sensing). The incident laser point was beamed at an angular tilt according to leaf orientation such that it was incident 13 cm perpendicular to at least six different points on the target flag leaf. The external probe component of the IRT was simultaneously used to record the ambient air temperature ( $T_e$ ). The difference ( $T_e - T_l$ ) was taken as the temperature depression (TD). For the field environments, each TD data point was the average of six readings per plant and 10 plants per RIL (five plants for each of the two replicates per RIL), including the plants that were randomly tagged for WL sampling. In the greenhouse, six readings were taken per plant for each of two plants per replicate. The TD readings were taken on the same day as the leaf wax but before leaf disks were taken. All the TD data were taken using the same IRT sensor to eliminate possible errors due to technical discrepancies between instruments. It should be noted that a population size of 100 RILs (and the two parents, for a

total of 102 lines) was determined to be optimal for the 2-h-constrained TD sampling window, the sample size, sampling instrument and sampling rate as defined for this experiment. The 100 RILs were used in QTL mapping.

#### Determining the phenotypic variance components, heritability and genetic correlation

To estimate the variance components, we applied residual maximum likelihood (REML) modeling tool in R version Ri 386 3.2.0 (R Core Team 2013). The REML method was chosen for its flexibility in analysis experiments involving fixed effects and random effects with no restriction to independent data, or to data with the same variance in any one stratum (O'Neill 2010; Hill et al. 2008). Environment, genotype and replicates were treated as random effects while WL and TD were the random response variables in the combined environment analysis. The resulting data were used to partition the phenotypic variance of each trait into genotypic variance, variance due to environment and the variance due to genotype by environment interaction. We estimated broad-sense heritability ( $H^2$ ) on an entry mean basis (Bernardo 2002) for each trait in each environment. In each environment, we also determined the proportion of phenotypic correlation between WL and TD that is attributed to genetic causes (a measure of their genetic correlation,  $r_g$ ) (Maes et al. 2004) using the phenotypic variance and heritability components according to the model below (Falconer et al. 1996):

$$r_g = \frac{r_p - e_1 e_2 r_e}{h_1 h_2} \text{ and } r_e = \frac{\sigma_{e12}^2}{\sigma_{e1}^2 \sigma_{e2}^2},$$

where  $r_p$  is the phenotypic correlation between trait 1 (WL) and trait 2 (TD), derived as  $r_p = h_1 h_2 \cdot r_g + e_1 e_2 \cdot r_e$ ;  $h_1$  and  $h_2$  are the broad-sense heritability of trait 1 and trait 2, respectively;  $e$  is the environmental variance and  $r_e$  is the non-zero environmental correlation between trait 1 and 2. The symbol  $\delta^2$  is the environmental variance on the traits.

The ratio of expected phenotypic correlation ( $r_p$ ) to that of the observed  $r_p$  (Wachs and Plomin 1991) estimated the proportion of the observed phenotypic correlation between WL and TD that is due to genetics. Observed phenotypic correlation between WL and TD was derived from the coefficients of

determination ( $r^2$ ) between the two traits in each environment. Expected  $r_p$  was determined as a bivariate heritability, calculated as the square roots of heritability of the two traits multiplied by their genetic correlation ( $\sqrt{h_1} \cdot \sqrt{h_2} \cdot r_g$ ), without the environmental correlation terms (Wachs and Plomin 1991; Waite and Levin 1998).

#### Statistical transformation for QTL analysis

Fluctuations in air temperatures within a sampling window were noted to be significant on some days. To reduce the error of variation due to this, the TDs were moderated following recommendations in previous studies (Keener and Kircher 1983; Reynolds et al. 1994, 1998; Wilman et al. 1992; Scott and Knott 1974). First, the minimum and the maximum ambient air temperatures were noted for each sampling day during the 12:00–14:00 sampling window. This data was used to cluster the recorded air temperatures ( $T_e$ ) within a day's sampling window into bin widths of 0.5 °C. The mean TD for each bin (cluster) was noted. The mean TD from 6 readings per plant was first divided by the mean TD of the representative cluster and then multiplied by the mean TD per plant to obtain a corrected TD value for the plant. This ensured minimal deviation from the actual raw TD, while achieving the versatility required to reduce the error variance (Kaufman and Rousseeuw 2009) associated with instantaneous ambient temperature fluctuations within a day's data collection window. The corrected TD correlated positively ( $r = 0.94$ ) with the raw TD data, and was applied in the subsequent regression analyses. Log-transform values of WL and the raw data for number of days to 50% flowering (FT) were also used. The effect of FT is reported only in the QTL analysis.

The homogeneity of phenotypic variance among locations was determined using the Lavene Test as modified by Brown–Forsythe (Ott and Longnecker 2010), and the normality assumption was checked by the Shapiro–Wilk Test (Ott and Longnecker 2010). A generalized linear model (GLM) was used to assess the model fitness and the coefficient of determination ( $r^2$ ) of TD as explained by WL. For the non-QTL analysis, the mean separation was determined using Fisher's LSD. Regression fits were performed in JMP Pro 12 (SAS) and bar graphs in MS Excel.

## QTL mapping and analysis

Digital genotyping (DG) (Morishige et al. 2013) was accomplished using the high throughput Illumina Genome Analyzer Platform II (Illumina.com 2014), a next generation sequencer previously described (Quail et al. 2001). The Illumina sequencer is hosted at the Texas A&M AgriLife Genomics and Bioinformatics Services in College Station, Texas, USA. The genotyping data was processed from BTx642/RTx7000 RIL population genomic DNA digested by *Ngo*MIV. A total of 886 DG markers were used in this QTL study. MapMaker/EXP version 3.0 (Stephen 1993) was used to create the genetic map.

The phenotypic values for WL and TD were averaged per RIL across reps to produce 100 sample values per trait in each location used for QTL analysis. WinQTL Cartographer V2.5 (Wang et al. 2011) was used to generate QTLs using composite interval mapping at a walk speed of 1 cM. For each trait, the data were permuted 1000 times to establish the QTL significance threshold. QTL above the 50th rank (95%) were deemed significant. QTL peak position, 1-LOD widths, and the proportion of phenotypic variance explained were extracted from the plots. All QTL were overlaid on the *Stg* QTL positions previously published (Crasta et al. 1999; Subudhi et al. 2000; Xu et al. 2000) for the same mapping population. These previously mapped *Stg* loci were derived from a visual phenotypic rating on a to 5 scale. A value of 1 corresponded no leaf senescence, and 5 represented total leaf senescence at grain maturity. Using similar scoring, other studies have confirmed and identified additional loci (Harris et al. 2007; Kassahun et al. 2010; Sanchez et al. 2002) for the staygreen trait in other regions of the sorghum genome. MapChart V2.2 (Voorrips 2002) graphical interface was used to organize the QTL bands on the linkage graphs obtained from WinQTL Cartographer V2.5.

### Identifying annotated genes under overlapping QTL for wax and QTL for staygreen

A primary interest in this study was the overlap of QTL for wax and staygreen; therefore, only the sequences in such regions of significant overlap were searched in detail. For the sake of this study, the marker coinciding with a QTL peak has been termed 'peak marker'. The digital genotyping (DG) QTL peak

markers at 1-LOD were taken to represent the position of the respective QTL. The range flanked by the vertical intersects to a QTL trace line (curve) on either side of the peak marker was taken as the QTL width. Each DG marker under a QTL is distinguished by a unique short genome sequence acquired from the polymorphic DG templates. The limits of the genomic sequence bound by a declared QTL were defined by the start nucleotide of the first marker and the end nucleotide of the last marker flanking a peak marker, depending on the read orientation (reverse or forward) of each marker sequence.

For QTL with distinct flanking markers, the genomic coordinates of the QTL were searched and viewed at the 'Pairwise Level' in the 'Genome Context' of the sorghum portal of the Plant Genome Database (PGDB), home browser SbGDB (*Sorghum bicolor* GDB) assembly version Sbi1/Sbi1.4/SbGDB181 (<http://plantgdb.org/SbGDB/>). Using the Zoom toggle in SbGDB Genome Context Tool, the subject chromosome was walked at 5 kb per toggle beginning with the 5' marker sequence until the end of the QTL boundaries. Annotated transcripts in this region were confirmed and noted. All sequences were downloaded in FASTA format.

Where an uncharacterized transcript was identified, its CDS (coding DNA sequence) was first queried in the NCBI's BLASTX (Basic Local Alignment and Search Tool for nucleotide to protein) (Stephen et al. 1997) context for orthologous protein translations. The search was refined in Phytozome V.10 (The Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute; [phytozome.jgi.doe.gov](http://phytozome.jgi.doe.gov)), BLASTX for the grasses node. The top eight BLASTX hits of non-redundant transcripts with small E-values below the threshold and scores greater than the threshold value were selected for multiple sequence alignment (MSA) in the UniProt platform (Consortium 2014) and their relatedness was ascertained through using CLUSTALW (Kyoto University Bioinformatics Center; <http://www.genome.jp/tools/clustalw/>) to construct a phylogenetic tree. The amino acid (AA) residue sequence of an annotated subject polypeptide which shared a phylogenetic node closest to the sorghum sequence was reported as the representative putative/characterized transcript. The annotation and gene ontology for this selected transcript were obtained in the UniProt Knowledge Base (UniProtKB/Swiss-Prot), a manually annotated and non-

redundant protein sequence database. The NCBI's DELTA-BLAST (domain enhanced lookup time accelerated-BLAST) was used for an improved identification of conserved base pair level functional domain, and the domain family tree noted in Pfam (Protein Families, <http://pfam.xfam.org/>) and InterPro of the European Molecular and Bioinformatics Lab, EMBL, <http://www.ebi.ac.uk/interpro>).

#### Ab initio prediction of the non-exomic function of non-annotated marker sequences

A marker sequence resident in an annotated transcript was noted as representing that transcript. In the case where no flanking markers were available or where a peak marker did not directly correspond to an exome region, the search was zoomed out to 1 kb immediately upstream and 1 kb immediately downstream of the peak marker sequences. The 1 kb region is within many of the new chromatin immunoprecipitation, ChIP-derived data of DNA segments containing non-exome functional elements (Worsley-Hunt et al. 2011). These were used in the first ab initio gene prediction in Genefinder in the FGENESH suit (Solovyev 2001; Solovyev et al. 2006; Yao et al. 2005) which applies the HMM (hidden Markov model) algorithm (Stratonovich 1960; Baum and Petrie 1966). HMM is used to find model parameters that best fit the observations given a model and empirical observations. Where no genes were predicted a second ab initio search was conducted for the discovery of *Cis*-acting regulatory modules in PlantCARE (Plant *Cis*-acting regulatory elements) (Magali Lescot et al. 2002) and confirmed in ESEfinder (exonic splicing enhancer finder) V3.0 (Smith et al. 2006; Cartegni et al. 2003).

## Results

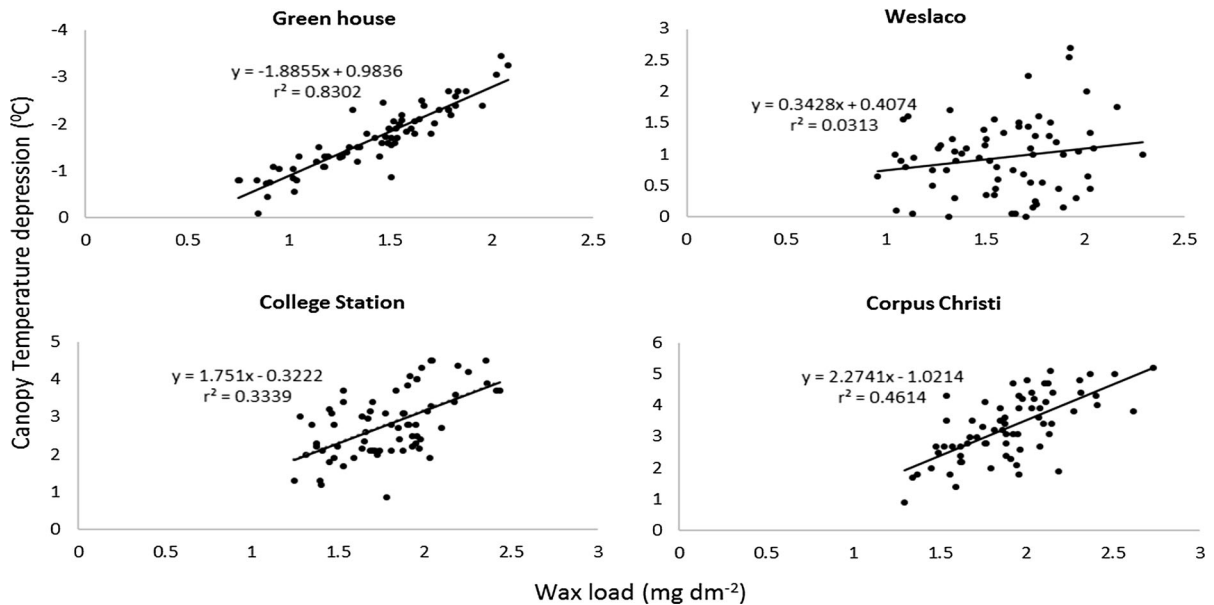
### Relationship between epicuticular wax load and plant canopy temperature

The mean air temperature recorded ~ 13 cm above the canopies and averaged for all the plots during flowering at College Station ranged between 41.5 and 46 °C, the highest among field environments. Corpus Christi had a mean of 39–41 °C and Weslaco was the lowest at 25–30 °C and comparable to the greenhouse

temperature of 24–26.5 °C. Among field environments, Corpus Christi had the highest correlation ( $r^2 = 0.46$ ) between WL and TD of the canopy (Fig. 1) with smaller correlations in College Station ( $r^2 = 0.33$ ) and Weslaco ( $r^2 = 0.03$ ). In comparison, the greenhouse conditions had the strongest correlation ( $r^2 = 0.83$ ). These results, in part, support previous findings (Mondal et al. 2015) that leaf wax load is correlated with the ability of a plant to keep itself cooler through increased albedo, resulting in reduced water loss under elevated heat stress.

We further tested this relationship by estimating the genetic correlations ( $r_g$ ) between WL and TD in the four environments. The strongest  $r_g$  (0.88) showed in the heat-stressed College Station, followed by Corpus Christi (drought-stressed,  $r_g = 0.79$ ), with the weakest genetic correlation ( $r_g = 0.17$ ) in the cooler Weslaco (Table 1). An  $r_g = 0.62$  was recorded between the two traits in the greenhouse. The proportion of the phenotypic correlation that was due to genetics was determined by the ratio of expected phenotypic correlation to the observed phenotypic correlation ( $\text{Exp } r_p / \text{Obs } r_p$ ), expressed as a percent. An estimated 57.1% of the  $\text{Obs } r_p$  in Corpus Christi was due to genetics, as were ~67% of  $\text{Obs } r_p$  in College Station and ~17%  $\text{Obs } r_p$  in Weslaco. Generally, across three of the four environments, the magnitude and trend of  $r_g$  did not correspond to those of  $\text{Obs } r_p$  but  $r_g$  corresponded near perfectly with  $\text{Exp } r_p / \text{Obs } r_p$ .

Broad-sense heritability ( $H^2$ ) within an environment ranged between 0.49 and 0.63 for WL and between 0.29 to 0.74 for TD, with the largest  $H^2$  being reported in the greenhouse environment for both traits. In combined analysis of all study environments,  $H^2$  of WL was 0.65 while  $H^2$  of TD fell to 0.11 (Table 1). There were no statistical differences between the mean WL in the Corpus Christi and the mean WL in College Station; mean WL were significantly larger in both environments, compared to those in the cooler, irrigated greenhouse and Weslaco (Table 1). The variance components for genotype (G) and genotype  $\times$  environment (G $\times$ E) were significantly higher than zero (the value zero was not included within 95% lower confidence limits and 95% upper confidence limit) for both WL and TD (Table 1). The environment variance component was surprisingly not significant for WL and for TD, suggesting that the differences in the phenotypic means and in heritability across environments, and also in the phenotypic



**Fig. 1** Regression relationship between canopy temperature depression (TD) and epicuticular wax load (WL) in each location for the RIL population. On the Y-axis is TD adjusted for transient ambient air temperature ( $T_c$ ) within a measurement

window (12:00–14:00). The canopy temperatures of all plants sampled in the greenhouse were warmer than the surrounding air temperatures, hence the negative sign on TD (vertical axis)

correlation between WL and TD across the environments, were influenced mainly by genotype and G×E interactions.

#### Pleiotropic QTL for wax

A total of 28 QTL on 5 chromosomes were identified for canopy temperature depression (TD), epicuticular wax load (WL) and flowering time (FT). Most of the loci alleles associated with high values for wax and TD were contributed by the pre-anthesis drought tolerant parent, Tx7000. Alleles for extended flowering time were mainly contributed by the senescence resistant (staygreen) parent, Tx642. In the combined analysis 16 QTL were detected for TD, 9 QTL for WL and 3 QTL for FT (Tables 2, 3).

A number of QTL were overlapping for associated traits. On SBI-10, the QTL *TDwl-10-58* explained 11.1% of phenotypic variance (PVE) for TD and it coincided with two QTL for leaf wax *WLwl-10-58* (PVE = 12.6%) and *WLCx-10-58* (PVE = 13.2%), detected in Corpus Christi and Weslaco, respectively. These QTL were associated with the same marker, *c10B13413*, located at 108.2 cM. This suggests that the locus may be pleiotropic and conditional. A

possible linkage between QTL for temperature depression and flowering time were also detected on other segments of SBI-10. For example, the QTL for TD (*TDwl-10-33*) was associated with marker *c10B4064* at 62.6 cM (PVE = 19.6%) and co-located with QTL *FTcx-10-35* (also detected in a second environment) at marker *c10F4552* (61.4 cM). On SBI-08, a QTL for TD, *TDcx-8-15* (19.6% PVE) and a QTL for FT, *FTcx-8-15* (11.7% PVE), both centered at 55.8 cM, collocated with a QTL for wax, *WLCx-8-17* (11.5% PVE) positioned at 57.7 cM. These QTL were detected in the same environment (Corpus Christi), suggesting that a group of tightly linked genes at this locus affect these traits. On SBI-06, QTL for TD (*TDcs-6-14*) and one for WL (*WLCs-6-14*) were detected in the same field environment (College Station). Both were associated with the marker *c6B8118* located at position 46.8 cM. The PVE attributed to the QTL were 13.5 and 12.1%, respectively. This further suggests a pleiotropic interaction between QTL for these two traits.

The *Stg* QTL that we have reported on SBI-01 and SBI-03 (Fig. 2) are the only ones which collocated with the other traits in this study. We did not identify any additional overlaps between the traits in this study

**Table 1** Average phenotypic values and derived genetic relationships for F12 sorghum recombinant inbred line (RIL) progeny and their parents Tx642 and Tx7000

Environment	WL				TD				Relationship between WL and TD <sup>a</sup>							
	Parent		RIL		Parent		RIL		$r_g$	Obs $r_p$	Exp $r_p$	Exp $r_p$ (Obs $r_p$ )/%				
	Tx642	Tx7000	Mean	Range	SD	H <sup>2</sup>	Tx642	Tx7000					Mean	Range	SD	H <sup>2</sup>
Corpus Christi	6.44b	8.2a	6.88b	0.85–5.6	1.03	0.57	3.2b	4.7a	3.22b	3.46–12.3	0.99	0.42	0.79	0.68	0.39	57.1
College Station	7.63a	6.33b	6.12b	0.85–5.4	0.92	0.49	4.5a	2.7b	2.78b	3.32–11.3	0.97	0.39	0.88	0.57	0.39	67.0
Weslaco	6.17b	5.36c	5.36c	0.00–3.3	0.97	0.51	1.3c	0.55d	1.63c	2.18–10.3	0.64	0.29	0.08	0.17	0.029	17.1
Greenhouse	4.51d	4.75d	4.31d	0.05–3.45	1.9	0.63	1.7c	2.09bc	1.6c	2.13–8.01	0.69	0.74	0.62	0.91	0.42	47.0
			Var		SE			H <sup>2</sup>		Var		SE				H <sup>2</sup>
Combined Environments																
Genotype (G)			2.24*		0.35						0.13*			0.05		
Environment (E)			1.19		0.98						1.06			0.87		
G×E			0.99*		0.08						0.60*			0.05		
H <sup>2</sup>								0.65								0.11

The Range of scored phenotypes, the standard deviation (SD), broad-sense heritability (H<sup>2</sup>), and correlations are provided for RIL progeny. H<sup>2</sup> was calculated from expected means obtained by ANOVA for each trait in each environment, and from the variance components obtained by REML model in the combined environments. Wax load (WL) values of the parents and the averages of the RILs connected by the same letter within and across environments are not significantly different at  $\alpha = 0.05$ ; canopy temperature depression (TD) values of the parents and the averages of the RILs connected by the same letter within and across environments are not significantly different at  $\alpha = 0.05$ . n = 100 RILs and 2 parents

$r_g$  genetic correlation,  $r_p$  phenotypic correlation, Obs observed, Exp expected (see methods), Var variance, SE standard error

\* Significant at  $\alpha = 0.05$

<sup>a</sup> Only data from RILs (n = 100) used



**Table 2** QTL detected in BTx642/RTx7000 Mapping Population (n = 100) in the greenhouse

QTL <sup>a</sup>	Markers	Position	LOD score	Additive	R <sup>2</sup>	Positive allele	Environment detected
WLgh-3-86	c3F19429	131		0.1222	0.1049	Tx7000	Gh, Wl
TDgh-3-74	c3B17049	104.6	2.6504	-0.2204	0.0986	Tx642	Gh
TDgh-8-1	c8B168	4	2.8813	-0.2399	0.1132	Tx642	Gh
TDgh-8-4	c8F787	11	3.3425	-0.243	0.1136	Tx642	Gh
FTgh-1-29	c1B5373	32.6	4.2071	-0.2277	0.1403	Tx642	Gh
FTgh-10-35	c10F4552	61.4	7.7535	8.3466	0.1572	Tx7000	Gh, Cx

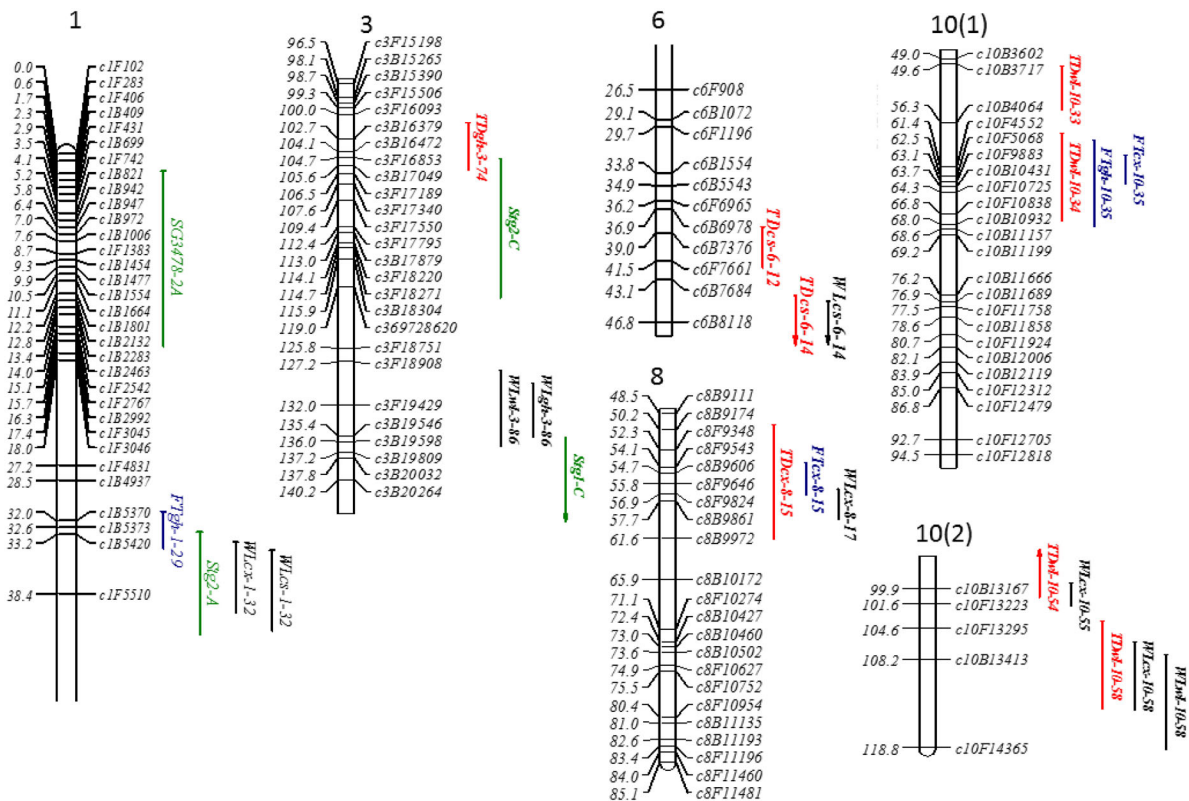
<sup>a</sup> QTL symbols: the first two upper case letters are the abbreviated traits (*WL* wax load, *TD* canopy temperature depression, *FT* flowering); the lower-case letters represent location (*gh* greenhouse); middle number is the chromosome number, and the last number in the marker number

Environment detected: Cx Corpus Christi, Gh greenhouse, Wl Weslaco

**Table 3** QTL detected in BTx642/RTx7000 Mapping Population (n = 100) in the field

QTL <sup>a</sup>	Markers	Position	LOD score	Additive	R <sup>2</sup>	Positive allele	Environment detected
Leaf cuticular wax							
WLcs-1-32	c1F5510	38.4	3.2668	-0.147	0.1293	Tx642	Cs, Cx
WLcs-6-14	c6B8118	47.2	3.4709	-0.1434	0.1208	Tx642	Cs
WLwl-3-86	c3F19429	131	3.3304	0.1218	0.1097	Tx7000	Wl, Gh,
WLwl-10-58	c10B13413	107	3.4668	0.126	0.1267	Tx7000	Wl, Cx
WLcx-8-17	c8B9861	57.7	3.1846	0.1219	0.1151	Tx7000	Cx, Wl
WLcx-10-50	c10B12119	83.9	2.6205	-0.1322	0.0917	Tx642	Cx
WLcx-10-55	c10B13167	99.9	2.8261	0.1401	0.1037	Tx7000	Cx
WLcx-10-58	c10B13413	108.2	3.6789	0.1397	0.1323	Tx7000	Cx, Wl
Temperature depression							
TDcs-4-67	c4B15449	124.4	3.086	-0.2987	0.095	Tx642	Cs
TDcs-6-12	c6F7661	41.5	3.156	-0.3039	0.103	Tx642	Cs
TDcs-6-14	c6B8118	47.2	4.0535	-0.3528	0.1353	Tx642	Cs
TDcs-8-8	c8F2049	42.3	4.8263	0.3807	0.1609	Tx7000	Cs
TDwl-10-33	c10B3717	52.6	4.3184	-0.3481	0.1654	Tx642	Wl
TDwl-10-34	c10B4064	60.3	5.5191	-0.3813	0.1967	Tx642	Wl
TDwl-10-42	c10B11157	68.6	3.3116	-0.278	0.1118	Tx642	Wl
TDwl-10-54	c10F12818	98.5	3.5743	0.2964	0.1308	Tx7000	Wl
TDwl-10-58	c10B13413	108.2	4.9324	0.3085	0.1578	Tx7000	Wl, Cx
TDcx-4-44	c4B13239	93.5	5.4034	0.4575	0.1647	Tx7000	Cx
TDcx-6-48	c6F12702	98.8	3.2203	-0.4713	0.1023	Tx642	Cx
TDcx-8-9	c8B7863	45	4.2133	0.454	0.1498	Tx7000	Cx
TDcx-8-15	c8F9646	55.8	6.2923	0.5053	0.1964	Tx7000	Cx
Flowering time (days to flowering)							
FTcx-8-15	c8B9861	55.8	3.0584	5.4434	0.1176	Tx7000	Cx
FTcx-10-35	c10F4552	61.4	3.4715	4.9952	0.0812	Tx7000	Cx, Gh

<sup>a</sup> QTL symbols: the first two upper case letters are the abbreviated traits (*WL* Wax load, *TD* canopy temperature depression, *FT* flowering); the lower-case letters represent location (*cs* College Station, *cx* Corpus Christi, *wl* Weslaco); middle number is the chromosome number, and the last number in the marker number. Environment detected: Cs College Station, Cx Corpus Christi, Gh greenhouse, Wl Weslaco



**Fig. 2** Graphical representation of linkage groups on chromosome segments. Segments of chromosomes 1, 3, 6, 8 and 10 are represented. The numbers on top of each center bar represents the chromosome number. Numbers to the left are genetic distances in cM. To the right are the QTL bars where the capital letters represent the trait identity: TD canopy temperature depression, FT flowering time, WL epicuticular wax load, and S staygreen. Mapping was done in QTL Cartographer V2.5 and the drawings and QTL bars organized in MapChart V2.2 graphical interface. The QTL for WL, TD and FT have been

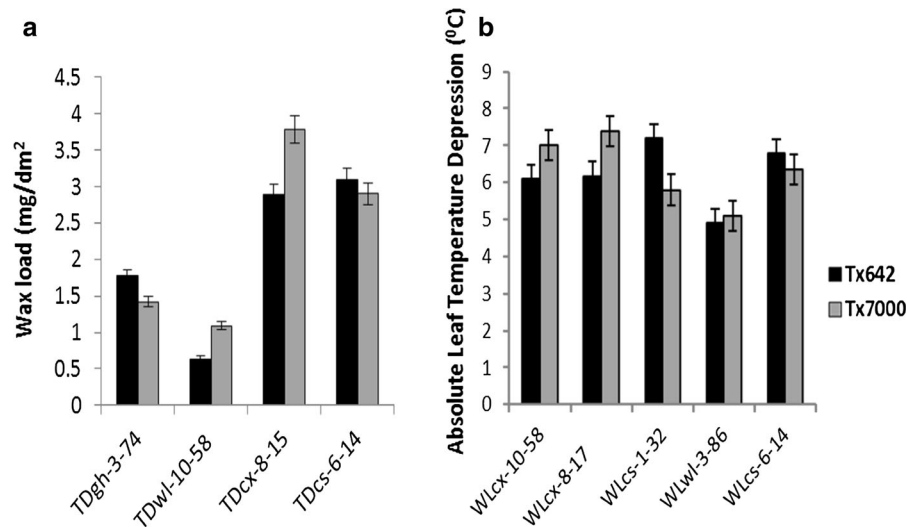
overlaid on Stg QTL previously mapped by others as cited in the methods. QTL symbols: the first two upper case letters are the abbreviated traits (WL Wax load, TD canopy temperature depression, FT flowering); the lower-case letters represent location (cs College Station, cx Corpus Christi, wl Weslaco); middle number is the chromosome number, and the last number in the marker number. Environment detected: Cs College Station, Cx Corpus Christi, Gh greenhouse, Wl Weslaco. n = 100 RILs from Tx642 X Tx7000

with the other *Stg* loci reported earlier. On SBI-01, we detected a QTL for leaf wax (*WLcs-1-32*) at 38.4 cM that shared the same marker position with *WLcx-1-32*, explaining 12.9% of the phenotypic variance. This QTL overlapped *Stg2-A* at marker *c1F5510* (Fig. 2). *Stg1-C*, centered at marker *c3B20264* at 140.2 cM (with interval 135.0–142.5 cM) on SBI-03 was in the same genomic region as two QTL for wax (*WLgh-3-86* and *WLwl-3-86*), centered at 135.4 cM.

On average, the mean wax and TD phenotypic contribution was increased by the Tx7000 allele with one exception (Fig. 3a, b). The QTL *WLcs-1-32* locus had the positive allele for wax from Tx642.

Annotated genes in the locus pleiotropic to wax, staygreen and flowering time

Two QTL for wax detected in two stress environments (College Station and Corpus Christi) coincided with *Stg2-A* and a QTL for flowering time. The genomic region bound by the overlapping QTL contained a gene cluster with a combined span of ~ 500 kb close to the centromeric region of chromosome 1 (1:13139511 to 13616615). The annotated genes identified in this locus (Fig. 3) include those that function in stress signaling and response (DRIP, CYP, MYB), photosynthesis and metabolite synthesis (CYP, MYB), transport (GGT, NSF) and reproduction



**Fig. 3** The mean genotypic effects of QTL for epicuticular wax load (WL, **a**) and QTL for canopy temperature depression (TD, **b**). Only a sample of the reported QTL that showed significant

overlap with those of temperature, wax or staygreen, have been shown. The parental sources of the alleles are identified by Tx642 and Tx7000.  $n = 100$

(FKL, CIPK4, KAN) (Table 4). This is a region of very low recombination, suggesting that the locus *WLcs-1-32* is likely inherited as a cluster of linked genes. A summary of selected genes in the other non-staygreen regions of QTL overlaps is shown in Table 2.

Analysis of the pleiotropic marker sequence and the flanking sequences

The 72 bp *cIF5510* marker is a multifunctional *Cis*-acting regulatory module consisting of core promoter and enhancer elements, and transcription factor elements known to regulate various stress-, light-responsive-, reproductive- and seed development-associated genes in various plants (Tables 5, 6). Notable elements include the CATT-motif, a ubiquitous *Cis*-acting element in plants, and the TATA-box, the core promoter motif in transcription. The CATT-motif was represented in *Zea mays*, while the TATA-box was represented in six plant species. On both strands (Table 5) is an RY *Cis*-motif, a promoter element which is conserved in many seed-specific promoters that control gene expression during late embryogenesis and seed development (Reidt et al. 2000).

Within 2 kb of the peak marker *cIF5510* is a putative plant-specific (Baranowskij et al. 1994) and multifunctional (Rose et al. 1999) MYB DNA-binding domain

(ID: IPR006447), and just ~500 bp upstream of the locus peak marker is a MYB binding motif (TAACTG) involved in drought-inducibility (Table 4). The flanking sequences (1 kb on both sides of *cIF5510*) consist of *Cis*-acting core regulatory elements in promoter and enhancer regions (TATA-box, TA-rich region, G-box and CAAT-box), light responsive (L-box, G-box), and Methyljasmonate, MeJA-responsiveness (CGTCA-motif) (Table 6). These observations suggest that the marker *cIF5510* represents a region that may be important in stress response and reproduction.

## Discussion

Wax and absolute temperature depression show a positive correlation which is both genotypically and environmentally dependent

In all the study environments, genotypes with a larger mean WL had a larger absolute TD than genotypes with smaller WL values. Mondal et al. (2015) also reported that under hot and dry conditions, leaf wax accumulation is positively correlated with the ability of a plant to keep itself cool. Stress may also have elicited a stronger wax response as a stress mitigation phenotype. Thus, large temperature may have combined with drought to elicit the stronger wax to TD

**Table 4** Genes in the pleiotropic loci

Marker	Individual QTL <sup>a</sup>	Putative transcript	Organism	Locus/domain/sequence ID or GenBank ID	Functional description of candidate genes
c1F5510	WLcs-1-32	Hypothetical protein	Sorghum	Sb01g014353.1; XP_002466751.1; GID8062257	Has a conserved domain (Pfam 13450), a NAD(P)-binding Rossmann-like domain. Binds nucleotides such as cofactors FAD, NAD <sup>+</sup> , NADP <sup>+</sup>
		Putative MYB DNA-binding domain superfamily protein (R2R3like Myb domain)	Maize and <i>Aegilops tauschii</i>	NP_001136686.1; EMT25059.1; IPR006447; TGRR01557	Regulates the expression of chalcone synthase, chalcone isomerase and dihydroflavonol reductase genes required for the biosynthesis of 3-deoxyflavonoids
		Glucosyl/glucuronosyl transferase	Sorghum	Sobic-001G164900.1	Transferase activity; transferring hexocyl groups; metabolism
		CYP87 (cytochrome P87)	Sorghum	Sb01g014340.1	Oxygen binding; monooxygenase; iron ion carrier; heme binding; electron carrier
		CYP450 (cytochrome P450)	Sorghum	XP_002464218; IPR001128	Heme binding in electron transfer
		Putative CYP450-like	Sorghum	NC_012870; XP_002464219	Heme binding in electron transfer
		KAN3	Sorghum	Sb01g014353.1	Regulate embryo pattering and modulate auxin flow during embryogenesis
		NSF( <i>N</i> -ethylmaleimide sensitive factor)	Sorghum	Sb01g014140.1	Required for vesicle-mediated transport; ATP binding
		CIPK4 (CBL-interacting protein kinase4)	Sorghum	Sb01g014090.1	Observed to induce high Ca <sup>+</sup> in maize pollen, self-incompatibility by inhibiting pollen tube growth in silk
		DRIP1	Sorghum	Sb01g014160.1	DREB2A—interacting protein 1 = ABA independent pathway
		FKL	Sorghum	Sb01g014196.1	FLK = flowering locus KH RNA binding domain
c3B17049	TDgh-3-74	UNKNOWN protein	Sorghum	Unidentified	EST, in oxidatively stressed leaves & roots
		ERGIC3 (endoplasmic reticulum-golgi intermediate compartment protein 3)	Oryza	LOC_Os03g57100.1	Possible role in transport between ER and Golgi
c3b19546	WLwl-3-86	CKX1 (cytokinin oxidase/hydrogenase)		Sb03g045410.1	Cytokinins shown to slow aging of plant organs—by preventing protein breakdown, activation of protein synthesis and assembly of nutrients from nearby tissues), also in plant pathogenesis by inducing resistance to
c6B8118	WLcs-6-14	Dof zinc finger domain-containing protein	Sorghum	Sb06g025680.1	Dof (=DNA binding with one finger) transcription factor proteins
c8F9646	FTex-8-15	Pseudouridine synthase family of proteins		Sb08g018120.1	A nucleoside in conjunction with aminoacyl transferases in tRNA; important in translation; also, possible protection against damage by radiation

**Table 4** continued

Marker	Individual QTL <sup>a</sup>	Putative transcript	Organism	Locus/domain/sequence ID or GenBank ID	Functional description of candidate genes
c8B9861	WLcx-8-17	RPB9-like	Sorghum	Sb08g018130.1	Putative DNA-directed RNA polymerase II subunit RPB9-like; transcription factor
c10F9883	TDwl-10-34	EST; UNKNOWN protein	Sorghum	Unidentified	EST, expressed as water stressed—WS1; oxidative stressed leaves and roots; ovary 1, embryo 1; ethylene treated seedlings; wounded leaves; P deficient

<sup>a</sup> Only one QTL symbol is given where two or more different QTL overlap

relationship in Corpus Christi ( $r^2 = 0.46$ ) compared to the other field conditions. However, under cooler temperatures, the relationship is less obvious. For example, there was no correlation in Weslaco but a large correlation in the greenhouse condition. Weslaco and the greenhouse are two substantially different environments and the variation in response may in fact be due to the differences in confounding environmental factors given the significant variances of genotype, and G×E interaction for both WL and TD (Table 1). However, additional evaluations are needed to fully dissect the factors involved.

Assessment of the genotypic correlation between WL and TD showed values ranging from 0.08 in the cooler, irrigated Weslaco field environment to a high of 0.88 in the heat-stressed College Station. This trend was very similar to the proportion of observed phenotypic correlation between the two traits that is due to genetics in each of the environments. The by-environment broad-sense heritability also showed a pattern that was similar between the two traits. These observations suggest that canopy temperature regulation is partly surrogate to the variation in WL and thus changing heritability of WL may also lead to corresponding changes in heritability of TD as a secondary, linked trait. Even though the magnitude and sign of phenotypic and genetic correlations observed may sometimes be unrelated (Réale and Roff 2001; Hill et al. 2008), our observations suggest that wax may play a role in conditioning canopy temperature and that WL has the potential to be used as an important phenotype for improving plant canopy-related stress tolerance goals.

Collocation of QTL for staygreen and those for leaf wax and temperature depression

Most *Stg* QTL are located on SBI-02 and SBI-05 (Xu et al. 2000; Haussmann et al. 2002; Subudhi et al. 2000; Crasta et al. 1999). In this study, only the staygreen QTL *Stg 1*, *Stg2*, *Stg3* and *Stg4* were considered as *Stg* loci (Xu et al. 2000; Crasta et al. 1999). No QTL for WL, TD and flowering were detected on these chromosomes in this study. In fact, only staygreen QTL on SBI-01 (*Stg2*) and SBI-03 (*Stg1* and *Stg2*) co-located with the QTL for any of these traits have been reported in this study (Fig. 2).

Two QTL for wax *WLwl-3-86* and *WLgh-3-86* centered at 135.4 cM (marker *c3F19429*) shared a

**Table 5** Functional motifs identified within 1 kb of the genomic sequences flanking the marker *c1F5510* on SbChr1:13615615-13617686

Site name	Organism	Position <sup>a</sup>	Strand	Matrix score	Sequence	Function
4 cl-CMA2b	<i>Petroselinum crispum</i>	1387	+	10	TCTCACCAACC	Light responsive element
5UTR Py-rich stretch	<i>Lycopersicon esculentum</i>	989	-	13	TTTCTCTCTCTCTC	<i>Cis</i> -acting element conferring high transcription levels
ABRE	<i>Oryza sativa</i>	1232; 1432	-	9	GCCGCGTGGC	<i>Cis</i> -acting element involved in the abscisic acid responsiveness
ATGCAAAAT motif	<i>Oryza sativa</i>	545	+	8	ATACAAAT	<i>Cis</i> -acting regulatory element associated to the TGAGTCA motif
Box 4	<i>Petroselinum crispum</i>	19	+	6	ATTAAT	Part of a conserved DNA module involved in light responsiveness
CAAT-box	<i>Hordeum vulgare</i>	6; 507; 718; 749; 810; 898; 1078; 1226; 1464	+	4	CAAT	Common <i>cis</i> -acting element in promoter and enhancer regions
	<i>Brassica rapa</i>	206; 531; 548; 644; 807	+	5	CAAAAT	Common <i>cis</i> -acting element in promoter and enhancer regions
	<i>Brassica juncea</i>	642	+	9	TGCAAAATCT	Common <i>cis</i> -acting element in promoter and enhancer regions
	<i>Glycine max</i>	744; 809; 816; 897;	+	5	CAATT	Common <i>cis</i> -acting element in promoter and enhancer regions
	<i>Arabidopsis thaliana</i>	1225	+	5	CCAAAT	Common <i>cis</i> -acting element in promoter and enhancer regions
CATT-motif	<i>Zea mays</i>	454	+	6	GCATTC	Part of a light responsive element
CGTCA-motif	<i>Hordeum vulgare</i>	500	+	5	CGTCA	<i>Cis</i> -acting regulatory element involved in the MeJA-responsiveness
G-Box	<i>Pisum sativum</i>	122	-	10	CACACATGGAA	<i>Cis</i> -acting regulatory element involved in light responsiveness
	<i>Zea mays</i>	332	+	9	GACATGTGGT	<i>Cis</i> -acting regulatory element involved in light responsiveness
GAG-motif	<i>Arabidopsis thaliana</i>	1142	-	7	AGAGAGT	Part of a light responsive element
GATA-motif	<i>Arabidopsis thaliana</i>	610	-	7	GATAGGA	Part of a light responsive element
GCN4_motif	<i>Oryza sativa</i>	1036	-	7	TGAGTCA	<i>Cis</i> -regulatory element involved in endosperm expression
HD-Zip 1	<i>Arabidopsis thaliana</i>	744	+	8.5	CAAT(A/T)ATTG	Element involved in differentiation of the palisade mesophyll cells
HD-Zip 2	<i>Arabidopsis thaliana</i>	744	+	8	CAAT(G/C)ATTG	Element involved in the control of leaf morphology development
HSE	<i>Brassica oleracea</i>	777	+	9	AGAAAAATTCG	<i>Cis</i> -acting element involved in heat stress responsiveness
L-box	<i>Petroselinum crispum</i>	1387	+	10	TCTCACCAACC	Part of a light responsive element
MBS	<i>Arabidopsis thaliana</i>	1105; 1321	-	6	TAACTG	MYB binding site involved in drought-inducibility
O2-site	<i>Zea mays</i>	873	+	9	GATGATGTGG	<i>Cis</i> -acting regulatory element involved in zein metabolism regulation

Table 5 continued

Site name	Organism	Position <sup>a</sup>	Strand	Matrix score	Sequence	Function
RY-element	<i>Helianthus annuus</i>	464; 626	+	8	CATGCATG	<i>Cis</i> -acting regulatory element involved in seed-specific regulation
Skn-1_motif	<i>Oryza sativa</i>	1447	-	5	GTTCAT	<i>Cis</i> -acting regulatory element required for endosperm expression
Sp1	<i>Oryza sativa</i>	366	-	6	GGGCGG	Light responsive element
TA-rich region	<i>Nicotiana tabacum</i>	60; 64; 62; 66	+	20	TATATATATAT ATATATATATA	Enhancer
TATA-box	<i>Arabidopsis thaliana</i>	16-34; 62-84; 141; 143; 152; 167; 201; 218; 220; 220-265; 478; 488; 552; 595-599; 664; 759-837; 956; 1148; 1187-1191; 1467; 1492	+	6	TATA	Core promoter element around -30 of transcription start
	<i>Glycine max</i>	18; 28; 31; 563	-	6	TAATA	Core promoter element around -30 of transcription start
	<i>Antirrhinum majus</i>	22	-	8	TATAAATT	Core promoter element around -30 of transcription start
	<i>Zea mays</i>	703	-	8	TTTTAAAA	Core promoter element around -30 of transcription start
	<i>Lycopersicon esculentum</i>	278; 704; 734; 1109	+	5	TTTTA	Core promoter element around -30 of transcription start
	<i>Brassica napus</i>	32; 63-83; 142; 485; 551; 594; 596; 795; 798; 1190; 1146	+	6	ATTATA	Core promoter element around -30 of transcription start
	<i>Pisum sativum</i>	245; 485	-	8	TATAAAAAT	Core promoter element around -30 of transcription start
TCT-motif	<i>Arabidopsis thaliana</i>	862	-	6	TCTTAC	Part of a light responsive element
TGA-element	<i>Brassica oleracea</i>	1088	+	6	AACGAC	Auxin-responsive element
TGACG-motif	<i>Hordeum vulgare</i>	500	-	5	TGACG	<i>Cis</i> -acting regulatory element involved in the MeJA-responsiveness

Motifs identified and analyzed in PlantCARE

Sequences downloaded from SbGDB and includes the 72 bp of the cIF5510 marker sequence

<sup>a</sup> Refers to the position in the sequence SbChr1:13615615-13617686 as represented in Table 6, counting from 5' end for the leading strand and 3' end for the complementary strand





**Table 6** continued

Strand	Sequence
>	ISBgenome SbChr1:13615615-13617686
+	TGGTCTGGTGGTCGATCGAGCCGAGAGATCGATCGAGAGGATCTCTACTCGACTACTA ACTGCTGCCTGCTGTGTGTCTGGGACTGGGACTGA TCTGGTGCCGGTACCTAGCTAGCTAGCTAACTCTCACCAATCCCCA
-	ACCAGACCACAGCTAGCTCGGCTCTCTAGCTAGCTCTCTAGAGATAGAGATGCTGATGATTGACGACGCACACACGACACACAGACCCCTGACCCCTGAC TAGACCACGGCCATGG ATCGATCGATTGAGAGTTGGTAGGGGT
+	CTACGTACGGATCGGAGCTAGCTAATGGATGCCACCGCACTCATCCATGACACGAAAGCACCGCAATAATGCAAGTGCCTGAAGGAGAACTATA GCTA
-	GATGCATGCCTAGCCTCGGATCGATTACCTACGGTGGGTGAGTAGGTA <b>CTGTGCTTCGTGCTGTATATACGTTACCGCACTTCCTTGTGATATCGAT</b>

1 kb on either side of the marker *cJF5510* sequence (bold). The actual frame length shown is shorter by ~560 bp on the 5' end that overlaps the centromere region. All the *Cis*-acting regulatory elements are shown in Table 5

1 AT-rich region (enhancer)

2 CATT-motif (light-responsive element)

3 RY-element

4,6 TATA-box—core promoter elements

5 TGACG-motif (*Cis*-regulatory element associated with MeJA-responsiveness)

7 HSE—Heat stress-responsive element

8 5'UTR Py-rich stretch (*Cis*-acting element conferring high transcription levels)

9 MBS (MYB binding site)

0.5 cM region with a *Stg1* QTL (*Stg1-C*) at 140.2 cM (marker *c3B20264*) on SBI-03. However, the significant wax alleles were from the non-staygreen parent Tx7000, suggesting that wax accumulation at this locus is independent of *Stg1*. Among all the stable staygreen QTL, *Stg1* QTL has been reported as one of the most important in drought tolerance after *Stg2* (Craata et al. 1999; Sanchez et al. 2002; Xu et al. 2000).

The QTL for wax, *WLcs-6-14* (12% PVE) overlapped QTL for canopy temperature *TD-6-14* (13.5% PVE) on SBI-06. Both were detected in College Station and the alleles were from the staygreen parent Tx642. The Tx642 alleles for the QTL *WLcs-6-14* was associated with a slightly larger but not significantly different wax load value compared to the contribution by Tx7000 alleles. A similar observation was made for the QTL *TDcs-6-14* for canopy temperature depression. However, the QTL *WLcs-1-32* (12.9% PVE) for wax load which overlapped the QTL for staygreen *Stg2-A* contributed to a significantly larger wax load phenotype associated with the Tx642 alleles compared the Tx7000 alleles. This QTL was mapped to a single marker *c1F5510* with another QTL for wax (*WLcx-1-32*) at 38.4 cM on the short arm of SBI-01 near the centromeric region. Both QTL were detected in both the high temperature-stressed but irrigated College Station and in the drought-treated Corpus Christi. These QTL were mapped very close to a QTL (*FT-1-29*) detected in the greenhouse for flowering time. These observations suggest that wax load and the staygreen-associated regulatory factors may be pleiotropic.

Furthermore, the detection and collocation of the *Stg* loci with the genomic loci for wax may indicate that the *Stg* QTL overlap in time with those conferring wax-related tolerances at anthesis. This implies that that the expression of the staygreen trait may be initiated earlier than traditionally thought. Such an inference is strengthened by the conclusions based on a few recent reports on possible pre-anthesis staygreen-specific expressions (Johnson et al. 2015; Kasahun et al. 2010; Burke et al. 2010).

A tight gene cluster in the pleiotropic locus associated with wax and staygreen

Diverse genes associated with stress signaling and response (DRIP, CYP, MYB), photosynthesis and metabolite synthesis (CYP, MYB), transport (GGT,

NSF) and reproduction (FKL, CIPK4, KAN) form a gene cluster within ~500 kb region (Table 3) within the locus *WLcs-1-32*, close to the centromere (Fig. 2). This is a region of very low recombination which suggests this locus is inherited as a cluster of linked genes.

Members of the MYB family of proteins are known to intervene in many biological processes including reproductive processes, photosynthesis and stress response, modulation of root growth and development and salicylic acid biosynthesis (Lee and Suh 2015). In the locus *WLcs-1-32* resides an orthologue of a plant-specific *Myb* domain (InterPro ID: IPR006447) which is associated with transcription regulation of stress responsive genes (Rose et al. 1999; Baranowskij et al. 1994), leaf coloration (Gao et al. 2013), floral meristem differentiation (Moreau et al. 2016) and transcriptional activation (Baranowskij et al. 1994). Other studies have shown that a *Myb* family member, MYB94 for instance, is a transcription factor that induces both stomatal closure and cuticular wax biosynthesis by upregulating directly cuticular wax biosynthetic enzyme genes such as ketoacyl synthases (KAS), the bifunctional wax ester synthase/diacylglycerol acyltransferase (WSD1), alcohol forming fatty acyl-CoA reductase (FAR) and ECERIFERUM (CER1 and CER3) (Wang et al. 2014; Lee and Suh 2015). Lee and Suh (2015) have also shown that in drought-treated *Arabidopsis*, MYB96-1D and MYB96-1 upregulates 19 genes involved in wax biosynthesis, including a long-chain acyl-CoA synthase (LACS3) and in transport including an ABC transporter (ABCG11/WBC11/COF1). Others include lipid transfer proteins (LTP) (Seo and Park 2011). Additive effects were recently reported between MYB94 and MYB96 in the activation of cuticular wax biosynthesis in *Arabidopsis* (Lee et al. 2016). The presence of the a MYB binding site (MBS, TAACTG) involved in drought-inducibility and the Drought Responsive Element Binding, DREB2A-Interacting Protein (DRIP1) within *WLcs-1-32* suggests that this QTL may be involved in drought-related responses. Further, the locus contains a second major domain, the Rossmann-like NAD\_binding\_8 (PFAM13450) which is a structural motif in the hypothetical protein SORBIDRAFT\_01g014353 (orthologue Si037864 m.g) (Table 3), which binds nucleotides such as cofactors FAD, NAD + and NADP + that are important in electron transfer in photosystems. Together with the presence of other *Cis*-acting elements in the promoter and enhancer regions

(Table 6), we suggest that the locus *WLcs-1-32* may be important in the transcription of stress responsive genes, which may include those regulating wax, leaf pigmentation and photosynthetic processes.

Similarly, the pleiotropic effect of *WLcs-1-32* can also be attributed to the CYP family of proteins in this locus. For instance, the CYP87 is reported to directly be associated with oxygen and electron transfer by acting in heme binding and as iron carrier during photosynthesis (Werck-Reichhart et al. 2002), while Phytochrome450 (P450)-like proteins are involved in stress and defensive compounds signaling cascades such as for plant hormones and fatty acids, cutin synthesis and functional plant pigments (Werck-Reichhart et al. 2002; Degtyarenko 1995). A Cytochrome P450 family member, CYP704B2, has been shown to catalyze the  $\omega$ -hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice (Li et al. 2010). A related protein, CYP96A15, which is also upregulated by a *Myb* gene, is a functional orthologue of Mid-chain Alkane Hydroxylase (MAH1), which converts alkanes to 1<sup>o</sup> alcohols and 1<sup>o</sup> alcohols to ketones in the 1<sup>o</sup> alcohol pathway in wax biosynthesis (Seo and Park 2011). Other genes residing in the locus intervene directly in the reproductive processes (CIPKL4, KAN3, FKL), drought (DRIP1) and vesicular transport (NSF, GGT).

The marker *c1F5510*, together with its 2 kb flanking sequences, is a region of *Cis*-regulatory module (CRM). Transcriptional regulation is usually mediated by the interaction of multiple transcription factors (TFs) with their respective specific *Cis*-regulatory elements (CREs) in the CRM in the DNA (Niu et al. 2014). Taken together, we suggest that the locus *WLcs-1-32* may have a role in the transcriptional regulation of wax biosynthesis and functional staygreen, among other functions during inflorescence and anthesis in Sorghum under stress.

## Conclusion

This study investigated the genetic overlap between QTL for staygreen, epicuticular leaf wax and canopy temperature depression. We have also reported that field stress conditions may elicit a stronger genetic correlation between epicuticular wax load and canopy temperature depression than the unstressed field

conditions. Thus, selecting for wax load may improve the proportion of observed phenotypic correlation which is due to genetics. This observation is strengthened by the fact that a number of traits collocated to the same QTL. The results suggest a possible strong linkage between QTL for epicuticular leaf wax and canopy temperature depression and that wax load influences plant canopy temperature. QTL associated with flowering time were also detected. The overlap between a QTL for epicuticular leaf wax and a QTL for staygreen (*Stg2-A*) showed that the alleles for larger epicuticular wax load was from the post-anthesis staygreen parent Tx642. This was different from the other QTL for larger epicuticular wax load which were associated with the pre-anthesis drought tolerant Tx7000. The onset of expression of the different staygreen loci may vary with stress level. Our results suggest that the genes controlling the staygreen trait may be linked with those controlling leaf waxes to influence a plant response to drought and heat stress at the reproductive stage. Further studies of these major QTL are recommended.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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