EVALUATION AND QTL MAPPING OF LEAF COMPOSITION TRAITS ASSOCIATED WITH THE STAY-GREEN PHENOTYPE IN SORGHUM [*Sorghum bicolor* (L.) Moench]

A Dissertation

by

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ABSTRACT

Stay-green is an important agronomic trait in grain sorghum that produces higher, and more stable grain yields under drought stress post-anthesis. Historically, stay-green has been evaluated and selected for visually in the field with a rating system known as leaf and plant death rating (LPD). LPD ratings are notoriously difficult to replicate, and require many environments for evaluation, primarily due to uncontrollable rainfall events, but also field variability. There is a need for a quick and reliable screening method to identify stay-green sorghums. The objectives of this research are to evaluate the efficacy of two lab-based bioassays for identification of stay-green within a RIL population derived from BTx642/Tx7000 and a set of diverse breeding lines. Additionally, leaf sugar compounds and there role in reducing post-flowering drought stress is explored through genetic analysis.

Quantum efficiency (Fv/Fm) measurements made on leaf tissue from each RIL were evaluated for correlations with leaf and plant death (LPD) ratings. Results indicate that statistical differences between stay-green classes and non stay-green classes exist with this bioassay. The two RIL parents in this study, BTx642 and RTx7000, had differential responses in both LPD rating and quantum efficiency. The assay was much less robust in separating the RILs, possibly due to intermediate LPD ratings observed in most RILs and also from environmental effects.

Leaf dhurrin at anthesis was quantified for each RIL in this study and was found to be highly correlated with LPD. Environment was the largest source of variation, much greater than genotype or replication. The dhurrin assay was recommended for

QTL mapping based on a strong correlation with LPD, and a low CV (%) observed in the study.

A leaf dhurrin QTL (*Dhu1*) was discovered in this study on SBI01 using the BTx642/Tx7000 RIL population. *Dhu1* was highly heritable and explained a large percentage of the variation of leaf dhurrin in the population. *Dhu1* is aligned with genes involved in dhurrin biosynthesis on SBI01. *Dhu1* is also aligned with a previously un-identified stay-green QTL (*Stg5*) on SBI01, consistent with previous studies identifying an association between leaf dhurrin and stay-green.

A diverse set of ten breeding lines were also evaluated in four locations in 2014 for leaf dhurrin, leaf sugars and stay-green (LPD). Dhurrin was highly correlated with LPD rating from 2014 and all documented stay-green lines contained 2-3x higher dhurrin levels than senescent sorghums. Repeatability for dhurrin and LPD was very high and GxE effects were relatively small, indicating that selection for high dhurrin is a good predictor for stay-green and that high dhurrin lines rank similarly when compared to low dhurrin lines, regardless of the environment.

DEDICATION

This is for you, Dad.

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I would like to thank my committee chair, Dr. Bill Rooney, for his support, patience and outstanding instruction during my time at Texas A&M University. I look forward to many years of collaboration. I would also like to thank my committee members, Dr. John Mullet, Dr. Patricia Klein and Dr. Gary Peterson for their time and support. I also thank Dr. John Burke for giving me the opportunity to go to graduate school to fulfill my career dreams.

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CHAPTER I

INTRODUCTION

A drought is defined as a prolonged or chronic shortage of rainfall and drought can occur in high, as well as in low rainfall areas and is relative to long-term average rainfall patterns and evapotranspiration demands (Wilhite and Glantz, 1985). Drought stress is the limitation of water available for plant growth and it is the primary constraint to productivity in grain crops throughout the world. Drought stress can negatively affect crop growth at any stage, but the magnitude and effect of drought stress differs depending on plant species and development stage. As the world population continues to increase, it is important to identify crop species with multiple uses with the ability to yield under variable climatic conditions, including drought conditions. One example of a plant species that has been proven as a multi-use crop with inherent drought tolerance is sorghum [*Sorghum bicolor* (L.) Moench] (Rooney, 2004).

Sorghum is a C_4 cereal crop species utilized throughout many parts of the world as a food, feed, forage, and as a feedstock for biofuel production. It ranks fifth in importance for cereal crop species in the world (Doggett, 1988). The crop originated in Africa over 5000 years ago and is best known for its drought tolerance capabilities, especially compared to other cereal crops like maize (Rooney, 2004). Sorghum has primarily been cultivated in the harsh, semi-arid environments of sub-Saharan Africa, India, Australia, and the United States. Although sorghum has traditionally been grown in sub-optimal growing environments, the crop contains an abundance of genetic diversity that allows for high yields and adaptation in many growing environments.

The ability of a plant to maintain green leaf area at maturity (GLAM), also known as stay-green, has been used in sorghum breeding programs for many years as a field based indicator for post-flowering drought resistance (Borrell et al., 2000a). Multiple physiological differences between stay-green and non-stay-green genotypes have been postulated as the casual mechanisms for the stay-green trait. Examples of potential stay-green mechanisms include increased leaf nitrogen at anthesis, higher chlorophyll content, increased photosynthetic activity and leaf greenness, and a reduction in leaf area (Borrell et al., 2000a, 2000b; Borrell et al., 2014 Rosenow et al., 1983).

Stay-green has been identified as a positive trait of interest by breeders and producers because the ability to stay-green in post-flowering drought stress conditions has been associated with increased grain yields (Jordan et al., 2012). The stay-green phenotype has also been shown to increase resistance to charcoal rot, stalk collapse, and lodging. There is a perception by some breeders that while stay-green hybrids yield more under post-flowering drought conditions, they are less responsive to fully irrigated, optimal conditions. One explanation for lower yields in stay-green hybrids under optimum conditions could be that the sources of stay-green used in breeding programs are relatively narrow, potentially coming from only one or two genetic backgrounds. Since the sources of stay-green are limited, fewer elite combinations of seed parents and pollinators are identified. The inclusion of new stay-green breeding lines with different genetic backgrounds would increase genetic variance and potentially lead to more favorable hybrid combinations, and subsequently, higher yield potential.

The current methodology for identifying and selecting stay-green plants in the field is by leaf and plant death (LPD) ratings (Xu et al., 2000). LPD ratings are field-based, visual ratings of GLAM after a post-flowering drought stress. Field based LPD ratings are notoriously difficult to manage and require multiple test environments, spanning multiple years, to provide an accurate assessment of the stay-green phenotype. However, stay-green can be difficult to phenotype due to environmental variation; rainfall after anthesis and natural field variation can eliminate the stress and drastically affect the LPD ratings. There remains a need for a simple, quantitative assay that accurately identifies stay-green lines without needing to grow them under post-flowering drought conditions. To be useful, this assay needs to be reproducible, cheaper than field screening, and provide the ability to screen at early growth stages.

Two physiological plant assays developed within the Lubbock USDA-ARS sorghum improvement team were used in this study to validate their efficacy in identifying the stay-green phenotype in sorghum. Both assays have been published and validated on diverse sorghum lines differing in stay-green response (Burke et al. 2010; Burke et al. 2013).

A difference in quantum efficiency (Fv/Fm) between known stay-green and non-stay-green lines was first observed by Burke et al. (2010). The hypothesis of this bioassay is that stay-green genotypes with higher concentrations of sucrose in the leaves are better suited for the 30-minute temperature challenge than are the non-stay-green, low sucrose genotypes. Results from Burke et al. (2010) indicate that statistical differences between stay-green classes and non stay-green classes exist with this

bioassay. The quantum efficiency assay also correlates positively with sucrose concentrations within the leaf (Burke et al., 2010). Most importantly for this study, the two parents used for QTL mapping in this study, BTx642 and RTx7000, had differential responses in quantum efficiency.

Burke et al. (2013) also found that historically known stay-green lines contained elevated levels of leaf dhurrin in comparison with non stay-green lines. Although the testing was done on a diverse set of breeding lines and not a structured population (i.e. RILs), a strong association between dhurrin and stay-green was found. Breeding lines with different genetic sources of stay-green, spanning across different sorghum working groups, all showed elevated leaf dhurrin concentrations. Although the exact mechanisms conditioning the stay-green phenotype are not fully understood, it is interesting to postulate that dhurrin in the leaves could act as an osmoprotectant and nitrogen storage molecule (Busk and Møller, 2002; Borrell and Hammer, 2000). Borrell and Hammer (2000) reported that leaf nitrogen concentrations at anthesis were higher in stay-green lines than in non-stay-green lines. These elevated levels of N in the leaves could be indirect measurements of dhurrin within the leaves, or N signaling differences due to dhurrin assimilation. Borrell et al. (2000a) specifically mentions a "missing piece" in relation to the previous work on stay-green causes and mechanisms. Dhurrin could be the "missing puzzle piece" that has eluded sorghum researchers for many years. This bioassay for stay-green identification is recommended for additional study due to the observed differences between BTx642 and RTx7000 by Burke et al. (2013), and because

the method has been shown to differentiate stay-green lines across varying environmental conditions.

Thus, the objectives of the research project are:

1. Evaluate the efficacy of the two bioassays developed by Burke et al. (2010; 2013) to discriminate between stay-green and non-stay-green lines within the BTx642/Tx7000 stay-green RIL population.
2. Map QTL for leaf dhurrin and determine if they align with known stay-green QTL.
3. Determine if identified QTL for dhurrin accumulation from the dhurrin assay align with the known genes in the dhurrin biosynthetic pathway.
4. Evaluate known stay-green and non stay-green breeding lines from different genetic backgrounds for differences in dhurrin accumulation and stay-green response.

CHAPTER II

ANALYSIS OF QUANTUM EFFICIENCY (Fv/Fm) AS A SCREENING TOOL FOR THE STAY-GREEN PHENOTYPE IN *Sorghum bicolor*

Introduction

Sorghum bicolor is a C_4 cereal crop species utilized throughout many parts of the world as a food, feed, forage, and as a feedstock for biofuel production. It ranks fifth in importance for cereal crop species in the world (Doggett, 1988). The crop originated in Africa over 5000 years ago and is best known for its drought tolerance capabilities, especially compared to other cereal crops like maize (Rooney, 2004). Sorghum has primarily been cultivated in the harsh, semi-arid environments of sub-Saharan Africa, India, Australia, and the United States. Sorghum contains an abundance of genetic diversity that allows for adaptation across a wide range of environments and high yields in those that are favorable.

Of all the factors that limit the productivity of sorghum, drought is the most important (Boyer, 1982). Stay-green has been used in sorghum breeding programs for many years as a field-based indicator of post-flowering drought resistance (Borrell et al., 2000a). Many studies have identified stay-green lines and also physiological differences between stay-green and non stay-green lines (Borrell et al., 2000a; 2000b; Rosenow et al., 1983).

Stay-green has been identified as a positive trait because the ability to remain green and viable during post-anthesis drought stress has consistently been associated with increased and more stable grain yields (Jordan et al., 2012). Stay-green is also

associated with resistance to charcoal rot, as well as resistance to late-season lodging (Rosenow et al., 1983). Jordan et al. (2012) identified a strong positive relationship between stay-green and grain yield under drought conditions, but positive associations were reduced and offset by negative associations in some environments under optimum growing conditions. There are several possible explanations for this observation. First, lower yields in stay-green hybrids in some non-stress environments could be due to unknown factors associated with stay-green such as the fact that stay-green genotypes hold greater concentrations of sugar and nitrogen in the stalk (Borrell et al., 2001). Also, since sources of stay-green are limited, fewer elite combinations of seed parents and pollinators are identified. The inclusion of new stay-green breeding lines, especially different from the widely utilized stay-green line BTx642, could increase genetic variance within a breeding program and theoretically could increase grain yields.

The current methodology for identifying and selecting stay-green plants in the field is by leaf and plant death (LPD) ratings (Xu et al., 2000a). LPD ratings are field-based, visual ratings of stay-green taken after physiological maturity. Field based LPD ratings have historically been difficult to reproduce, usually requiring multiple test environments and multiple years to provide a reliable stay-green evaluation. This inconsistency in phenotyping is due to environmental variation such as rainfall after anthesis, which eliminates the stress and differences among genotypes for LPD ratings. Thus, a simple quantitative assay that accurately identifies stay-green lines regardless of the environment is needed. To be useful, this assay needs to be reproducible and cost-effective.

Burke et al. (2010) first described a lab based stay-green screening assay that evaluated final quantum efficiency (Fv/Fm) after a 30-minute temperature challenge at 40°C as a prediction tool for stay-green. The assay utilized an Opti-Science OS1-FL Modulated Fluorometer to measure low levels of unknown drought protection systems produced naturally in the plant. Examples of these systems include many sugar compounds that are hypothesized to allow for osmoregulation within the plant (Burke et al., 2010). Burke et al. (2010) hypothesized that genetic differences for these protection systems exist and sorghum lines would respond differently based on their individual drought protection systems. Results from the assay indicate that stay-green lines contained more osmoregulation compounds and these compounds allowed for stay-green plants to produce higher final Fv/Fm values after temperature stress. Specifically, the highly studied stay-green line BTx642 produced higher final Fv/Fm values than the non stay-green line Tx7000. Although differences between known stay-green and non stay-green classes were observed in Burke et al. (2010), no specific correlation data between final Fv/Fm and stay-green rating was reported. There is a need to evaluate this novel stay-green assay in multiple environments and to compare stay-green ratings with final Fv/Fm.

The objectives of this study are to analyze genotype, environment, and GxE effects for Fv/Fm within the BTx642/Tx7000 RIL population. Additionally, correlations between stay-green and Fv/Fm are assessed and heritability (h^2) for Fv/Fm is estimated on an entry-mean basis.

Materials and Methods

RIL Population and Experimental Design

The RIL population BTx642/RTx7000 consisting of 97 F_{12} lines was used in this study. BTx642, also known has B35, was derived from a BC_1 selection of BTx406 and IS12555 (a durra sorghum) and has excellent post-flowering (stay-green) drought tolerance (Borrell et al., 2000a). Tx7000, also known as Caprock, is a historically important restorer line that is pre-flowering drought tolerant, highly susceptible to post-flowering drought stress, and when originally released was high yielding as a cultivar *per se (*Rooney, personal communication). The performance of Tx7000 represents the drought response common of many temperately adapted germplasm in U.S. breeding programs. In addition to the RILs, near-isogenic lines (NILs) of Tx7000 that contain previously identified stay-green QTL are also available (Harris et al., 2007). The RIL population, parents and NILs have been studied extensively for traits associated with the stay-green phenotype in sorghum (Xu et al. 2000a; Harris et al., 2007; Borrell et al., 2014).

The parents, RILs, and NILs were grown in replicated field trials in six environments between 2009 and 2011 to estimate quantum efficiency (Fv/Fm). These environments were designated as CS09, LB09, LB10, LB11, CS11, and WE11 (Table 1). College Station (CS) is located in south-central Texas and is a sub-tropical environment. Weslaco (WE) is located in the Rio Grande Valley of south Texas and is a humid, but semi-arid sub-tropical environment. Lubbock (LB) is located on the High Plains of Texas and has a semi-arid temperate climate. In all locations, the populations

and parents were planted in a randomized complete block design (RCBD) with two replications.

Table 1. Description of the environments in which BTx642/Tx7000 recombinant inbred lines were evaluated for stay-green or Fv/Fm.

Year	Location	Env	Trait evaluated
1993	Chillicothe, TX	(CD93)	Stay-green
1994	Lubbock, TX	(LD94)	Stay-green
1997	Lubbock, TX	(LL97)	Stay-green
1998	Halfway, TX	(HL98)	Stay-green
2009	College Station, TX	(CS09)	Fv/Fm
2009	Lubbock, TX	(LB09)	Fv/Fm
2010	Lubbock, TX	(LB10)	Fv/Fm
2011	College Station, TX	(CS11)	Fv/Fm
2011	Lubbock, TX	(LB11)	Fv/Fm
2011	Weslaco, TX	(WE11)	Fv/Fm

Agronomic Practices

Sorghum seeds were planted at a rate of 70 seed per plot at a depth of 4 cm and a plot in each environment was defined as a single row 5.2 meters long. Row spacing in each environment was 1.0 m wide except for the College Station location, which was 0.76 m. The Weslaco and College Station environments were furrow irrigated as needed to minimize visual drought stress. The Lubbock environments were drip irrigated from underground drip lines at a rate of 6 mm of water a day. All seeds were treated with a

seed treatment mixture of Captan®, Concept® and Gaucho® to allow for the application of Dual herbicide for pre-germination weed control.

Stay-Green Phenotyping

The parents and 97 RILs have previously been evaluated by Xu et al. (2000b) under post-flowering drought stress conditions in between 1993 and 1998 and that stay-green data is used herein. These environments were designated as CD93, LD94, LL97 and HL98; which represent Chillicothe (CD), Texas, Lubbock (LL, LD), Texas and Halfway (HL) Texas and are all production environments that commonly experience post-flowering drought stress. These environments and stay-green (LPD) ratings from Xu et al. (2000b) were used because the full irrigation requirements of the Fv/Fm bioassay did not produce environments in 2009-2011 where differences in stay-green response could be expressed.

Chillicothe is located in north-central Texas and is a semi-arid, temperate environment that consistently experiences temperatures above 33°C, especially later in the growing season. Halfway, like Lubbock, is a semi-arid, temperate environment located on the High Plains of Texas. Plots were defined as 4.9 m long and 1.0 m wide. In all locations, the experimental design was a randomized complete block design with three replications per entry. Plots were irrigated to avoid any drought stress until anthesis at which irrigation was terminated to allow post-flowering drought stress to develop later in the growing season. Average high temperatures for the period of study were 33°C with low relative humidity, conditions that are typical of West Texas and many Mediterranean climates where rainfall late in the season is unreliable. Because late-

season rainfall can drastically minimize visual stay-green differences in the field, only locations that expressed visual differences were recorded.

Stay-green was visually scored on the parents and RIL individuals on a plot basis by Xu et al. (2000b) using a scale of 1 to 5 based on the degree of visual plant greenness or senescence after physiological maturity. A rating of 1 indicated a completely green plant with green leaves and no senescence. A rating of 5 corresponded to no visual greenness in the leaves accompanied with complete plant death.

Quantum Efficiency (Fv/Fm) Assay

Quantum efficiency was determined according to the procedure described by Burke et al. (2010) in the environments grown in 2009 and 2011. At sunrise in each environment, five leaf punches were taken from representative plants within a plot using a number 6 cork borer and rubber stopper. The leaf punches were taken from plants between 60-70 days after planting. The punches were taken on the newest fully expanded leaf, excluding the flag leaf. The punches were transferred to a well in a Costar 3524 24-well cluster (Corning Inc, Corning, NY) that contained water filled to half capacity. The container lid was immediately returned to the well plate to ensure tissue samples did not dry out while in the field.

After returning to the lab, the leaf punches were arranged on a moistened Model 583 Gel Dryer Filter Paper (Bio-Rad Laboratories, Hercules, CA) in a Pyrex dish. The leaf punches and paper were covered with CO_2 permeable Glad Cling Wrap and rolled flat with a speedball roller to remove any air bubbles trapped underneath the wrap. Quantum efficiency (Fv/Fm) was measured on the tissue samples at the start of the

experiment using an Opti-Science OS1-FL Modulated Fluorometer. After initial sampling, the dish containing the samples was placed in the dark in an incubator set at 40°C. The samples were temperature challenged for 30 minutes within the incubator. After 30 minutes, the samples were removed from the incubator and allowed to temperature equilibrate at room temperature (25°C) for 30 minutes. The leaf tissues were then re-sampled after the temperature challenge for quantum efficiency (Fv/Fm) and the obtained Fv/Fm value after the temperature challenged was analyzed and is presented herein.

Statistical Analysis

Individual data on stay-green response in the form of LPD rating, and final Fv/Fm from each environment was analyzed to determine if variances were homogeneous. Heterogeneity of error variances was not detected so a combined analysis was performed. The statistical model used for the combined analysis was

$$\textit{Dependent Variable} = \mu + \textit{Genotype(G)} + \textit{Environment(E)} + \textit{GxE} + \textit{Rep[Env]} + \textit{Error}.$$

JMP® Version 10.0.0 was used for all statistical analysis. All sources of variation were considered random effects. Multivariate analysis was performed using Pearson correlations. Coefficient of variation (CV) was calculated according to Bernardo (2010). Because these RILs were fully inbred, a narrow-sense heritability (h^2) estimate for final quantum efficiency (Fv/Fm) on an entry-mean basis was calculated as:

$$h^2 = \sigma^2_{G} / [\sigma^2_{G} + (\sigma^2_{GxE/E}) + (\sigma^2_{error/ER})]$$

where σ^2_G is the variance due to genotype, σ^2_{GxE} is the variance due to the interaction of genotype and environment, $\sigma^2_{(error)}$ is the variance due to experimental error, *R* represents the number of replications within a given environment, and E is the number of environments.

Results and Discussion

Proportionally, the effect due to genotypic was the largest, accounting for 15.8% of the variation (Table 2). Residual error accounted for 62.4% of the variation, and was the largest source of variation in this study. A combined heritability (h^2) for quantum efficiency (Fv/Fm) was 0.69 across six environments.

Table 2. Variance components and narrow-sense heritability for quantum efficiency (Fv/Fm) from combined data from the BTx642/Tx7000 RIL population.

Source of Variation	Variance Component Estimate	Percent Variation	Probability > F
Environment (E)	0.001	5.6	0.219
Rep[E]	0.0021	11.7	0.001***
Genotype (G)	0.0028	15.8	0.001***
GxE	0.0007	4.3	0.074
Error	0.0113	62.4	
R^2	0.44		
CV (%)	28		
h^2	0.69		

The final quantum efficiency (Fv/Fm) value of BTx642 was higher in all environments as well as the combined analysis although the differences were more pronounced in some environments (Table 3). BTx642 was statistically higher for final Fv/Fm than Tx7000 in every environment tested (Table 3). Burke et al. (2010) also observed differences between BTx642 and Tx7000 with mean Fv/Fm values of 0.49 and 0.26, respectfully. In addition to the parents, of the four Tx7000 NILs containing previously identified stay-green QTL, only *Stg3* NIL was statistically higher than Tx7000 in LB10 and LB11 environments and had a combined final Fv/Fm value of 0.42 (Table 3). Although *Stg3* NIL produced a higher mean final Fv/Fm value of 0.42, it was only 85% of BTx642.

Table 3. Combined LS means for Fv/Fm efficiency for the RIL parents and Stg NILs in six environments from 2009 to 2011.

Environment	BTx642	Tx7000	Stg1 NIL	Stg2 NIL	Stg3 NIL	Stg4 NIL	$LSD_{p<.05}$
CS09	0.51	0.34	0.39	0.39	0.44	0.39	0.12
LB09	0.56	0.38	0.42	0.31	0.45	0.48	0.26
LB10	0.38	0.29	0.19	0.20	0.44	0.26	0.14
CS11	0.48	0.29	0.36	0.29	0.29	0.31	0.11
LB11	0.42	0.26	0.29	0.27	0.51	0.35	0.19
WE11	0.60	0.36	0.27	0.21	0.27	0.30	0.14
Mean	0.50	0.31	0.32	0.28	0.40	0.35	0.14

Table 4. Summary statistics for Fv/Fm for the BTx642/Tx7000 RIL population grown in six environments between 2009 and 2011.

Environment	Minimum	Maximum	Mean	Standard deviation
CS09	0.28	0.58	0.42	0.09
LB09	0.17	0.66	0.43	0.2
LB10	0.16	0.60	0.34	0.17
CS11	0.20	0.52	0.34	0.11
LB11	0.13	0.71	0.36	0.23
WE11	0.17	0.67	0.34	0.13

Combined across all environments, final Fv/Fm values did not significantly correlate with visual stay-green ratings in the BTx642/Tx700 RIL population (Table 5). Within individual environments, only the final Fv/Fm values in CS09 produced statistically significant correlations with stay-green ratings in the LD94 and HL98 environments and these were relatively weak relationships (Table 5). There was no correlation in the combined analysis.

Table 5. Pearson correlations between quantum efficiency (Fv/Fm) and visual stay-green within specific environments using the BTx642/RTx7000 RIL population.

Environment	LB09	CS09	LB10	LB11	WE11	CS11
CD93	-0.11	-0.18	-0.02	0.00	0.03	-0.05
LD94	-0.13	**-0.21***	0.01	0.00	0.00	0.01
LL97	0.13	-0.05	0.06	0.01	0.07	0.10
HL98	-0.02	**-0.24***	-0.07	0.00	0.00	-0.11

*Significant at 0.10

Although no significant GxE interaction was observed in this study, the individual environments used to study Fv/Fm values did appear to separate into two classes based on whether the environment was drip irrigated daily, or was furrow irrigated periodically throughout the growing season (Figure 1). The drip-irrigated environments produced a more bimodal distribution with higher variance and more separation between RILs while the furrow-irrigated environments had a normal distribution with less variance and less separation between RILs (Figure 1).

Figure 1. (A) Observed bimodal frequency distributions for final quantum efficiency (Fv/Fm) in continuous drip irrigation environments.
(B) Observed normal frequency distributions for quantum efficiency (Fv/Fm) in periodic furrow irrigation environments.

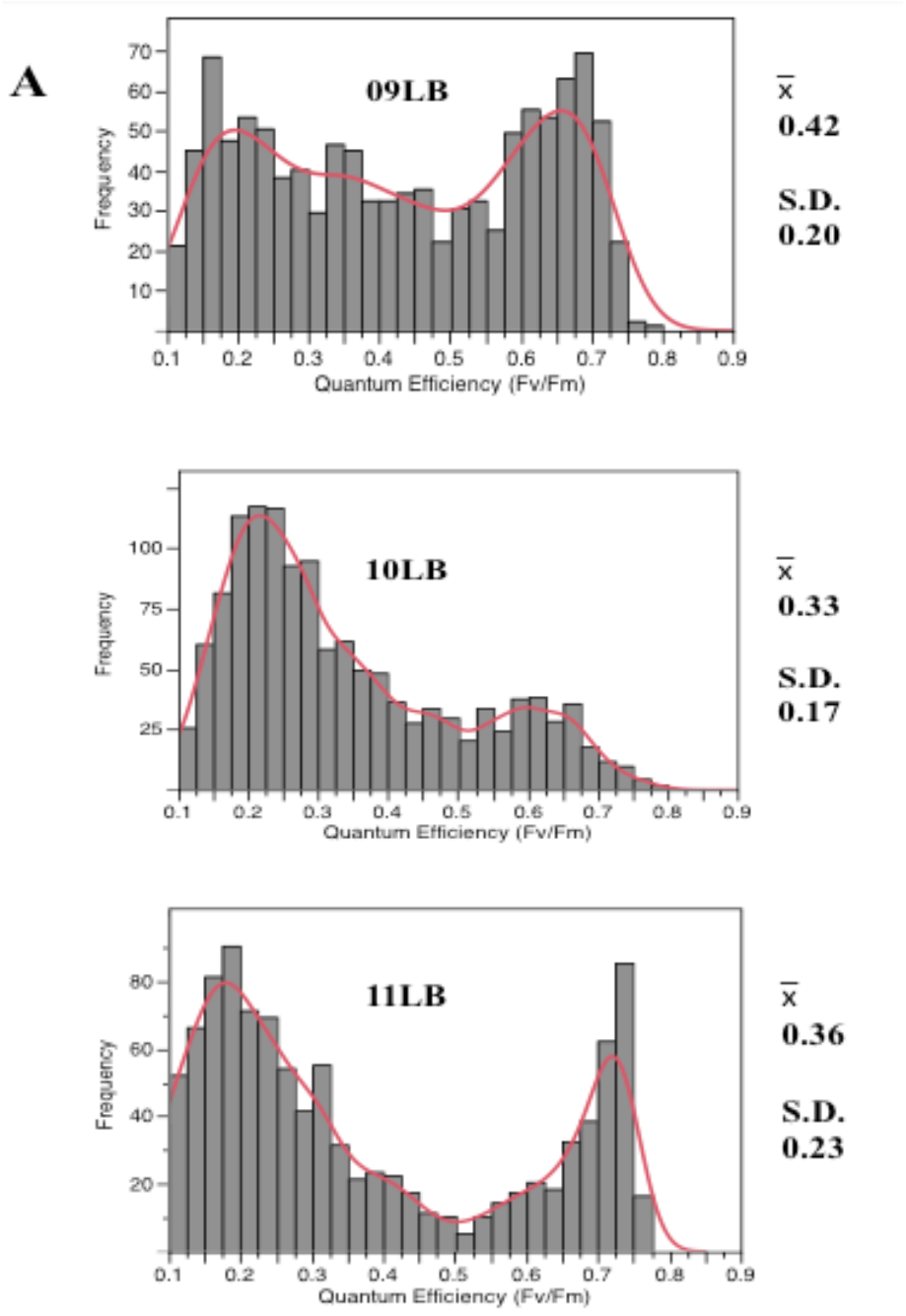
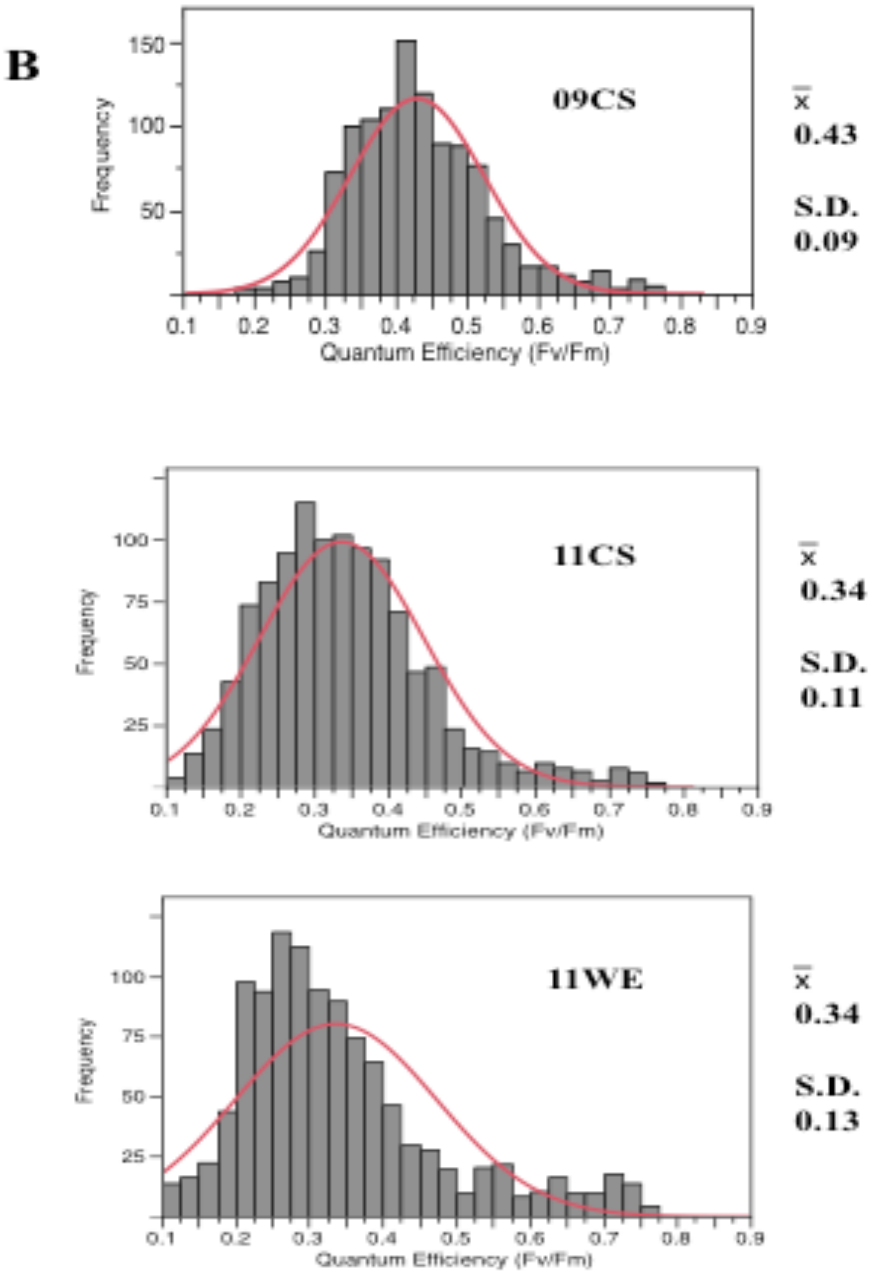


Figure 1 (Cont.)

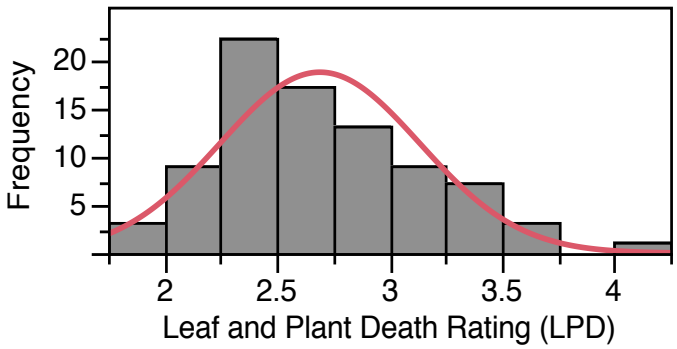


The germplasm used in this study was a set of RIL parents with significant range for stay-green (Table 6). The majority of the RILs themselves had stay-green ratings between the parents and contained much less stay-green variation than did the breeding lines originally studied in Burke et al. (2010). The average stay-green rating for the RILs was 2.97, with 75% of RILs averaging between 2.36 and 2.98 (Figure 2).

Table 6. Phenotypic ratings for BTx642, Tx7000, and RIL's for stay-green rating (LPD) in four environments. Data adopted from Xu et al. (2000b).

Category	LPD rating
BTx642 mean	2.28
Tx7000 mean	4.73
RIL mean	2.97
RIL range	1.23 - 5.00
CV (%)	16.62

Figure 2. Frequency distribution of the average stay-green rating (LPD) for the BTx642/Tx7000 RIL population in four environments (CD93, LD94, LL97 and HL98).



Quantiles	LPD rating
Maximum 100%	**4.02**
Quartile 75%	**2.98**
Median 50%	**2.64**
Quartile 25%	**2.36**
Minimum 0%	**1.91**

Results from this study confirm that the Fv/Fm bioassay effectively discriminates between established and definitively stay-green and non-stay-green lines that have significantly different stay-green ratings. Although the bioassay did separate the parents in this study, it did not accurately correlate with stay-green ratings in the RIL population. Previous reports on this assay tested established lines that were either highly stay-green or not stay-green (Burke et al., 2010). Results herein, using a range of genotypes with intermediate phenotypes for stay-green, indicate that the Fv/Fm assay is not robust enough to distinguish between these intermediate levels of stay-green. Additionally,

individual RILs that ranked highest for final Fv/Fm in one environment did not necessarily rank similarly in other environments (Table 7).

Table 7. Final Fv/Fm LS means for the five highest RILs and the RIL parents in six environments from 2009 to 2011. Parentheses indicate rank order from highest final Fv/Fm to lowest within each environment.

Genotype	Environment					
	09LB	09CS	10LB	11LB	11WE	11CS
6_8_Stgn	0.47 (9)	0.52 (3)	0.37 (15)	0.36 (10)	0.53 (1)	0.40 (11)
4_6_	0.46 (14)	0.43 (43)	0.34 (40)	0.37 (7)	0.42 (11)	0.40 (12)
78_80_	0.47 (6)	0.46 (26)	0.35 (28)	0.37 (2)	0.32 (56)	0.40 (9)
34_37_	0.45 (30)	0.52 (2)	0.36 (20)	0.36 (23)	0.41 (15)	0.41 (5)
55_58_	0.48 (1)	0.45 (36)	0.38 (5)	0.36 (9)	0.30 (71)	0.30 (13)
BTx642	0.46 (11)	0.50 (11)	0.35 (37)	0.36 (33)	0.50 (3)	0.42 (3)
Tx7000	0.42 (75)	0.37 (96)	0.31 (70)	0.35 (73)	0.35 (37)	0.31 (79)

The absence of a correlation between final Fv/Fm and stay-green and a non-significant GxE interaction can possibly be explained by variability, presumably due to the bioassay. This was very likely in the CS and WE environments where plants were more drought-stressed compared to LB. The CS and WE environments were furrow (flood) irrigated at specific times to maintain growth and eliminate drought stress. Thus moisture was not consistently available as was the situation under drip irrigation. It is likely that the variable irrigation pattern, including mild drought stress, created the environmental differences and more random (normal) distributions of final Fv/Fm

observed in (Figure 1). However, when these environments were removed from analysis, the correlation between Fv/Fm and stay-green improved only minimally (-0.05 to -.11) indicating that even under ideal irrigation conditions the assay may have difficulty in separating lines with intermediate and similar stay-green ratings. The relatively high CV value observed in this study also implies that random error comprised a large percentage of the variation observed, as confirmed in the analysis of variance (Table 2). The inability to effectively predict stay-green in the BTx642/Tx7000 RILs indicates that the bioassay is sensitive to environmental variations and may not be reproducible, especially in environments not describe by Burke et al. (2010).

Conclusion

The Fv/Fm bioassay was originally developed to determine water-deficit stress in cotton (Burke, 2007). Burke et al. (2010) reiterated that the bioassay be used on well-watered sorghum to identify the stay-green response. The LB environments used in this study were dripped irrigated at a rate of 6 mm of water a day, received a daily fertilizer application via the drip system, and represented these types of environments. Although no significant correlation for stay-green and Fv/Fm was observed in any of the environments, all environments did effectively separate BTx642 and Tx7000 into two classes of germplasm but the RILs themselves failed to separate consistently into distinct classes.

This assay is effective at identifying the phenotypic extremes of stay-green, proven by the confirmed separation of BTx642 and Tx7000. Reliable identification of

phenotypic extremes for stay-green response is highly valuable in breeding programs where a quick screening method is needed for evaluation of many individuals in a short amount of time. The Fv/Fm assay appears to be much less robust is identifying minor differences in stay-green like observed in the BTx642/Tx7000 RIL population. More study is needed to evaluate the efficacy of this assay to accurately predict stay-green, especially in lines with an unknown or intermediate stay-green response.

CHAPTER III

EVALUATION OF THE CYANOGENIC GLUCOSIDE DHURRIN AS A BIOMARKER FOR THE STAY-GREEN PHENOTYPE IN *Sorghum bicolor*

Introduction

Dhurrin [(S)-p-hydroxymandelonitrile-B-D-glucopyranoside] is a cyanogenic glucoside that is produced by sorghum species, primarily in the leaves but also in stems and roots (Rhykerd and Johnson, 2007). Dhurrin is stored in the vacuoles of plant cells and is a bound, non-volatile precursor to the toxin hydrogen cyanide (HCN). HCN is known to be a highly toxic gas and is postulated as a plant defense response against herbivores (Krothapalli et al., 2013). Dhurrin catabolism in the form of HCN has been observed to be highly heritable (0.89-0.95) on an entry-mean basis (Eck et al., 1975).

The biochemical pathway of dhurrin is well understood, with known biosynthetic and catabolic genes available in the literature (Kahn et al., 1997; Møller, 2010, Hayes et al., 2015). The biosynthesis of dhurrin begins with the amino acid tyrosine and two membrane-bound P450 enzymes, CYP79A1 and CYP71E1. The final step involves UGT85B1, a glycolsyltransferase enzyme to produce dhurrin. Dhurrin has also been proposed as an available source of nitrogen, and as an osmoprotectant when a plant is exposed to limited water (Busk and Møller, 2002; Burke et al., 2015).

Stay-green is an important drought tolerance trait that delays plant senescence and allows for photosynthetic activity to continue late in the growing season when grain development is occurring. Stay-green is associated with increased charcoal rot resistance, a reduction in lodging, a stability of seed size, and increased grain yields

under drought conditions post-anthesis (Rosenow and Clark, 1995, Jordan et al., 2012). Production environments with enhanced water holding capabilities that receive early season rainfall but consistently turn dry late in the season are conditions ideal for stay-green sorghums. Many casual mechanisms have been postulated to help produce the stay-green phenotype in grain sorghum including a reduction in leaf size, a reduction in tillering, and modified root architecture (Borrell et al., 2014).

Burke et al. (2013) also observed that known stay-green lines contained elevated leaf dhurrin levels. The lines evaluated by Burke et al. (2013), represent different working groups of sorghum, as well as different maturities. A strong association between dhurrin and stay-green was observed in that study but there is a need for further investigation of the association in a structured genetic population (RILs) with multiple years of both stay-green phenotypes, and dhurrin concentration.

The objectives of this study were to analyze genotype, environment, and GxE effects for leaf dhurrin within the BTx642/Tx7000 RIL population; determine the correlations between stay-green ratings and leaf dhurrin content; and to estimate heritability (h^2) for leaf dhurrin is estimated on an entry-mean basis.

Materials and Methods

RIL Population and Experimental Design

The stay-green RIL population BTx642/Tx7000 was used for evaluation in this study. The population consists of 97 F_{12} RILs that have been extensively studied for traits associated with the stay-green phenotype in sorghum (Xu et al., 2000b; Harris,

2007; Borrell et al., 2014). BTx642 (B35) was derived from a BC_1 selection from the cross of BTx406 (donor parent) and IS12555 (recurrent parent) and is an excellent stay-green line. BTx642 was released by Texas A&M AgriLife Research in 2003. Tx7000 (Caprock) is a widely used restorer line that is a poor stay-green line but possesses excellent pre-flowering drought tolerance and produced high yields as a line *per se* when it was originally releases in the 1940's as a pure line cultivar (Rooney, personal communication).

The RILs and parents were grown in replicated field trials in four environments for leaf dhurrin analysis in 2013. The environments studied were designated as PR13, CS13, LB13, and TAES13 (Table 8).

Table 8. Description of the environments in which BTx642/Tx7000 recombinant inbred lines were evaluated for stay-green or leaf dhurrin.

Year	Location	Env	Trait evaluated
1993	Chillicothe, Tx	(CD93)	Stay-green
1994	Lubbock, Tx	(LD94)	Stay-green
1997	Lubbock, Tx	(LL97)	Stay-green
1998	Halfway, Tx	(HL98)	Stay-green
2013	College Station, Tx	(CS13)	Dhurrin
2013	Lubbock, Tx	(TAES13)	Dhurrin
2013	Lubbock, Tx	(USDA13)	Dhurrin
2013	Guayanilla, Pr	(PR13)	Dhurrin

Puerto Rico (PR) is a humid tropical environment with short days and is a common winter nursery location. College Station (CS) is a sub-tropical environment located in south-central Texas with a humid and hot growing season. The environments LB and TAES are locations on the High Plains of Texas near Lubbock with a dry, temperature climate. In all locations, a randomized complete block design with two replications per location was used. Seeds were sown at a rate of 70 seeds per plot at a depth of 4 cm using a John Deere MaxEmerge planter converted as a small plot research planter. A plot in each environment was defined as a single row 5.2 meters in length. The CS and TAES environments were furrow irrigated periodically throughout the growing season to minimize visual drought stress. The PR and USDA locations were drip irrigated and were considered fully irrigated. All seeds were treated with a seed treatment mixture of Caucho®, Concept®, and Captan® to allow for the application of pre-emergent herbicides and to protect against early insect infestation.

The parental lines and 97 RILs were also evaluated under post-flowering drought stress conditions in Chillicothe, Lubbock, and Halfway Texas in 1993, 1994, 1997, and 1998 by Xu et al. (2000b). Stay-green data from the environments described above from Xu et al. (2000b), and not from the 2013 environments, were used for this study because they represent the environments used to originally map stay-green QTL in sorghum and because the 2013 environments used for dhurrin analysis did not produce post-flowering drought stress where definitive stay-green phenotypes could be observed. Plant growth, management and stay-green phenotyping have been described in Xu et al. (2000b).

Dhurrin Assay

Dhurrin was extracted from the RILs near anthesis in each environment. For each plot, a single leaf punch measuring 1.0 cm in diameter was taken from five representative plants within each RIL plot for a total of five pooled samples per entry. Punches were collected from the youngest, fully expanded leaf, excluding the flag leaf. The leaf punches were taken midway between the blade tip and base and did not include the midrib. After collection, the leaf punches were immediately placed on ice in the field and were quickly returned to the laboratory. All HPLC analysis was performed at the Cropping Systems Research Laboratory (CSRL) in Lubbock, Texas. The tissue samples collected from the CS13 and PR13 environments were packaged in dry ice and shipped overnight to CSRL for analysis. Dhurrin from the five leaf punches was extracted in 1 mL 80% ethanol at 60°C for one hour followed by five minutes at room temperature. The extract was centrifuged for 10 minutes at 10,000 RPM and the resulting supernatant was transferred to an Eppendorf tube and dried with a vacuum centrifugation system (Savant Instruments Inc.) set on low drying. The extract was suspended in 200 µl of deionized water and separated on a YMC polyamine II column with a mobile phase of 75% acetonitrile (C_2H_3N) at a flow rate of 1.5 mL/min. Dhurrin was identified by its retention time in comparison with a standard and was expressed as $\mu g/cm^2$.

Statistical Analysis

Individual data from each environment was analyzed to determine if variances were homogeneous. Heterogeneity of error variances was not detected so a combined analysis was performed. The statistical model used for the combined analysis was

Dependent Variable = μ + *Genotype(G)* + *Environment(E)* + *GxE* + *Rep[Env]* + *Error*. JMP® Version 10.0.0 was used for all statistical analysis. All sources of variation were considered random effects. Multivariate analysis was performed using the Pearson method. Raw dhurrin data was log transformed to correct for ANOVA normality assumptions. Coefficient of variation (CV) was calculated according to Bernardo (2010). A combined narrow-sense heritability (h^2) estimated for leaf dhurrin was calculated as:

$$h^2 = \sigma^2_{G} / [\sigma^2_{G} + (\sigma^2_{GxE/E}) + (\sigma^2_{error/ER})]$$

where σ^2_G is the variance due to genotype, σ^2_{GxE} is the variance due to the interaction of genotype and environment, $\sigma^2_{(error)}$ is the variance due to experimental error, *R* represents the number of replications within a given environment, and E is the number of environments.

Results and Discussion

Of the four sources of variation partitioned in this experiment, genotype was the largest source of variation and accounted for a much greater proportion than environment, GxE, or replication (Table 9). The coefficient of variation (CV) for dhurrin concentration was relatively low at 10%, indicating random error represented a small percentage of the observed dhurrin concentrations. A combined heritability (h^2) for dhurrin content per unit leaf area was 0.89 across four environments.

Table 9. Variance components and narrow-sense heritability for leaf dhurrin content from combined data from the BTx642/Tx7000 RIL population.

Source of Variation	Variance Component Estimate	Percent Variation	Probability > F
Environment (E)	70.13	11.2	0.109
Rep[E]	13.28	2.1	0.003***
Genotype (G)	230.52	36.9	0.001***
GxE	94.34	15.1	0.001***
Error	216.68	34.7	
R^2	0.44		
CV (%)	28		
h^2	0.69		

The dhurrin concentration of BTx642 was higher than that of Tx7000 in the combined analysis and all four individual environments. Combined across all environments, BTx642 averaged 57.1 μg/cm^2 and Tx7000 averaged 16.7 μg/cm^2 (Table 10). Burke et al. (2013) reported similar results with BTx642 containing 3-4x the dhurrin content of RTx7000. In addition to the parents, the four NILs of Tx7000 described by Harris et al. (2007) containing previously identified stay-green QTL were evaluated for dhurrin. All NILs were statistically similar to Tx7000 with average dhurrin contents near 16 μg/cm^2. Within in the RIL population, leaf dhurrin concentration ranged widely and transgressive segregation was observed. The minimum mean dhurrin concentration was 8.7 μg/cm^2, and the maximum mean dhurrin concentration was 118.8 μg/cm^2 (Table 10.). The mean dhurrin concentration combined across all four environments was 37.1 μg/cm^2, which is approximately intermediate of the RIL parents BTx642 and Tx7000.

Table 10. Summary means for leaf dhurrin (μg/cm^2) within the BTx642/Tx7000 RIL population grown in four environments in 2013.

Environment	Parents			RIL Population			
	BTx642	Tx7000	$LSD_{p<.05}$	Min.	Max.	Mean	S.E.M.
PR13	61.4	20.1	36.4	8.7	93.1	31.1	1.9
CS13	50.5	16.1	30.0	9.4	113.8	48.4	2.6
USDA13	43.8	15.3	15.8	10.2	89.9	27.6	1.2
AgriLife13	73.4	14.8	34.4	11.8	118.8	39.1	1.8
Mean	57.1	16.7	18.9				

Stay-green phenotypic means and ranges for the BTx642/Tx7000 RIL population were previously described in Chapter II.

Across all environments, there was a strong and significant correlation between dhurrin concentrations and visual stay-green ratings (r= -.59). Comparing individual environments, leaf dhurrin was correlated with stay-green in all combinations with a minimum correlation of -0.51 and a high of -0.64 (Table 11).

Results from this study confirm that dhurrin effectively discriminates between stay-green and non-stay-green lines. The dhurrin bioassay did consistently separate the parents in this study and individual RILs that ranked highest for dhurrin ranked similarly in other environments (Table 12).

Table 11. Correlations between leaf dhurrin concentrations (μg/cm^2) and visual stay-green within specific environments for the BTx642/RTx7000 RIL population.

Environment	TAES13	PR13	USDA13	CS13
CD93	-0.55**	-0.54**	-0.59**	-0.63**
LD94	-0.59**	-0.57**	-0.59**	-0.64**
LL97	-0.53**	-0.56**	-0.60**	-0.63**
HL98	-0.51**	-0.60**	-0.60**	-0.63**

** Significant at 0.05

Table 12. Dhurrin LS means for the five highest RILs and the RIL parents in four environments in 2013. Parentheses indicate rank order from highest dhurrin to lowest within each environment.

Genotype	Environment			
	CS13	PR13	TAES13	USDA13
66_68_	70.4 (18)	47.7 (10)	84.6 (1)	61.5 (2)
18_21_Stgn	96.7 (5)	65.9 (1)	60.1 (3)	53.6 (3)
81_83_	104.9 (3)	32.6 (27)	55.0 (6)	74.7 (1)
53_56_Stgn	82.3 (7)	61.9 (2)	49.0 (12)	44.6 (5)
68_70_	103.7 (4)	50.0 (6)	54.3 (7)	31.7 (23)
BTx642	49.5 (36)	48.2 (9)	58.8 (5)	41.0 (8)
Tx7000	20.4 (68)	25.4 (46)	25.3 (78)	17.4 (65)

Burke et al. (2013) identified an association between high levels of leaf dhurrin and stay-green and the breeding lines evaluated were a diverse set of lines categorized as stay-green or non stay-green based on multiple publications. Results from this study confirm that dhurrin is highly effective at discriminating between known stay-green and non-stay-green RIL lines. In all environments evaluated, a high correlation was observed between leaf dhurrin and stay-green. Heritability (h^2) for leaf dhurrin was high in this study, indicating that breeding progress for leaf dhurrin can be made. Previously studies in sorghum have also identified cyanogenesis as a stable, highly heritable trait (Eck et al., 1975). This study expands on the investigation of cyanogensis and the production of HCN by identifying that like HCN-P, dhurrin biosynthesis is also highly heritable. The stay-green parent (BTx642) consistently contained 3-5x the dhurrin levels of the senescent parent (Tx7000) within this study.

The identification of an association between non-senescent (stay-green) grain sorghum lines and elevated HCN-p has been previously observed (McBee and Miller, 1980). Within that study, two stay-green (SC599, SC56) grain breeding lines had elevated HCN-p and were 3-7x higher than non-stay-green grain sorghum lines. There was also strong evidence that plant maturity and developmental growth stage influences overall dhurrin (HCN-p) concentration (McBee and Miller, 1980). The BTx642/TX7000 RIL population used is this study varies greatly for flowering date and physiological maturity with an average difference of two weeks between the earliest RIL and the latest RIL, depending on the environment (Rooney, personal communication). The tissue sampling for HPLC analysis was performed only once when the population was

approximately at anthesis. Differences in sampling time and maturity between the different environments could also help explain such a large environment effect. Another explanation for such a large environmental effect could be differential applications of nitrogen fertilizer. Nitrogen fertilizer has been known to induce dhurrin biosynthesis and HCN-p in sorghum plants beyond the seedling stage (Busk and Møller, 2002). Fertilizer was not controlled or standardized in this experiment, therefore, additional studies evaluating environmental effects of dhurrin accumulation in sorghum will need to control nitrogen fertilization, and also account for ambient N concentrations in the soil.

Acute or chronic exposure to HCN can lead to severe illness and even death in animals, including humans (Finnie et al., 2011). Exposures above 600 ppm of HCN have been documented as a potential threat level (Gleadow and Møller, 2014). Forage sorghums have been bred with low HCN-p specifically because animals graze the plant tissue, or the plant tissue is harvested and fed as fodder. Grain sorghum has not traditionally received HCN-p concerns because typically only the grain is harvested. A study by McBee and Miller (1980) did identify that two stay-green lines (SC599 and SC56) maintained elevated and potentially dangerous levels of HCN-P within the leaf tissue at harvest. Grain sorghums bred specifically for stay-green that contain high levels of leaf dhurrin may not be suitable as a dual-purpose forage crop due to the potential for HCN poisoning of livestock.

How leaf dhurrin conditions a stay-green phenotype in grain sorghum remains unclear. An endogenous turnover pathway for dhurrin that results in the formation of N-rich compounds ammonia and 4-hydroxphenylacetonitrile has been recently

hypothesized (Møller, 2010). It is possible that dhurrin serves an important function in primary metabolism as a transporter of nitrogen and glucose. Nitrogen dynamics and the mobilization of nitrogen have been observed previously to associate with the stay-green phenotype in grain sorghum (Borrell and Hammer, 2001).

The average cost of dhurrin biosynthesis has been estimated to be 2.1 g glucose per 1.0 g of dhurrin (Gershenzon, 1994). Secondary plant metabolites, which include defense compounds, have previously been associated with a metabolic cost at the expense of plant growth and reproduction (Neilson et al., 2013). Although plant metabolism has traditionally been separated into primary and secondary metabolism, these distinctions may be over-simplified. Extensive variation for leaf dhurrin exists within sorghum, including grain sorghum breeding lines used in many breeding programs (Burke et al., 2013; Hayes et al., 2015). Although there is an abundance of genotypic and phenotypic diversity among breeding lines, there is not an obvious negative phenotype associated with high leaf dhurrin under optimal growing conditions.

Investigation of the breeding lines used by Burke et al. (2013) to study dhurrin provides anecdotal evidence that grain sorghum breeding programs have selected against leaf dhurrin in the majority of their germplasm. Examples of breeding lines that are low in dhurrin and are included in many of the pedigrees of elite, high yield grain lines in the United States include SC170, SC1211, Tx7000 and BTx623. Additionally, breeding lines and hybrids from U.S. sorghum seed programs have been recently evaluated by USDA-ARS in Lubbock, TX and the vast majority of lines and hybrids were identified to be low dhurrin (Hayes, unpublished results).

Conclusion

Within the BTx642/Tx7000 RIL population, leaf dhurrin is strongly associated with the stay-green phenotype. Heritability (h^2) for leaf dhurrin is high, consistent with studies that identified HCN-p as highly heritable. QTL mapping of leaf dhurrin in BTx642/Tx7000 is recommended for additional study due to the consistent association between dhurrin and stay-green. A low CV and high heritability observed in this study provide evidence that QTL mapping could be beneficial in elucidating causal genetic mechanisms associated with dhurrin and stay-green. Additionally, investigation of the metabolic costs associated with high leaf dhurrin in breeding lines is needed to identify if the plasticity of secondary metabolites minimizes negative effects associated with dhurrin or if there is a yield penalty associated with the significant concentrations of leaf dhurrin.

CHAPTER IV

DISCOVERY OF A DHURRIN QTL IN SORGHUM: CO-LOCALIZATION OF DHURRIN BIOSYNTHESIS GENES AND A NOVEL STAY-GREEN QTL

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench, a C_4 grass, is a food, feed, forage, and biofuel crop that ranks fifth in world importance (Doggett, 1988). The species is known for its high biomass and grain yield potential, drought tolerance, and abundance of genetic variation (Mullet et al., 2014; Rooney, 2004; Rooney et al., 2007). Sorghum is primarily grown for grain production in drought prone regions in the Unites States, Africa, Australia, and India and is a staple cereal grain for millions of people throughout the developing world (Rooney, 2004). Drought stress can occur at any development stage and in grain sorghum, water limitation occurs at pre-flowering (G5 stage), or post-flowering (G9 stage), depending on location and climate. Physiological responses to water deficits that begin just before anthesis and continues into the grain-filling phase have been studied extensively (Borrell et al., 2000a; Harris et al., 2007; Rosenow et al., 1996; Tuinstra et al., 1998; Xu et al., 2000).

Post-flowering drought tolerance is an important mechanism that delays plant senescence and allows for greater retention of green leaf area during grain development. Stay-green has been a major focus of researchers working in water limiting climates that

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occur in Australia, the High Plains of the United States, and India where drought stress after anthesis is common in most production years. In terminal drought environments, stay-green is associated with reduced lodging, resistance to charcoal rot, and increased grain yield potential (Borrell et al., 2000a; Jordan et al., 2012; Borrell et al., 2014). Multiple sources of stay-green have been used in grain sorghum improvement programs for many years (Burke et al. 2013; Harris et al., 2007; Jordan et al., 2012; Reddy et al., 2007; Rosenow and Clark, 1981). In addition to breeding lines conveying the stay-green phenotype, many stay-green QTL have been reported (Borrell et al., 2014; Crasta et al., 1999; Harris et al., 2007; Subudhi et al., 2000; Tuinstra et al., 1997; Xu et al., 2000). Recently, *Stg1-4* were found to reduce tillering and leaf size, thereby reducing water use prior to anthesis and providing more water for crop development post-anthesis (Borrell et al., 2014a). Other stay-green QTL are aligned with QTL for root angle, indicating that more optimal distribution of root systems and water extraction potential could contribute to stay-green (Mace et al., 2011). Prior studies also identified a link between crop nitrogen content, extent of N-mobilization post anthesis and the onset of leaf senescence associated with differences in stay-green (Borrell and Hammer, 2000; Borrell, 2001; Thomas et al., 2002).

The cyanogenic glucoside dhurrin [(S)-*p*-hydroxymandelonitrile- β-D-glucopyranoside] is a defense allelochemical that can accumulate to high levels in sorghum leaves (Adewusi, 1990; Gleadow and Møller, 2014). Dhurrin is known to produce the toxin hydrogen cyanide (HCN) when leaves are crushed and is deemed an undesirable trait in forage (Rhykerd and Johnson, 2007). Although HCN is known to be

highly toxic and volatile, the metabolite dhurrin itself is sequestered within the vacuoles of plant cells and is non-toxic (Busk and Møller, 2002). Dhurrin has also been proposed to function in plant defense against insect herbivory, and as a storage source of nitrogen with osmoprotectant properties (Burke et al., 2015; Busk and Møller, 2002; Krothapalli et al., 2013). Recently, Burke et al. (2013) demonstrated a correlation between genotypes with elevated leaf dhurrin levels before flowering and genotypes with higher stay-green rating.

The dhurrin biosynthetic pathway is well documented in sorghum (Gleadow and Møller, 2014). Genes encoding enzymes involved in the synthesis of dhurrin (CYP79A1, CYP71E1, UGT85B1) are clustered on the distal arm of chromosome 1 (SBI01). Two genes encoding dhurrinases, enzymes that mediate the first step in catabolism, are present on chromosome 8 (SBI08). Hayes et al. (2015) identified extensive variation for dhurrin content within a large set of sorghum conversion (SC) lines and using GWAS, found significant associations between variation in leaf dhurrin levels and regions of SBI01 and SBI08 respectfully, encoding enzymes for dhurrin synthesis and turnover.

In this study, it is tested whether there is a genetic basis for the correlation between elevated dhurrin levels and stay-green phenotypes observed by Burke et al. (2013). Prior studies showed that N-uptake, total plant N content and mobilization play a critical role in expression of the stay-green phenotype in sorghum (Borrell et al., 2001; Borrell and Hammer, 2002; van Oosterom et al., 2010a). Dhurrin ($C_{14}H_{17}N0_7$) catabolism releases NH_3 that could be assimilated, providing a source of N in water

limiting conditions (Gleadow and Møller, 2014). While main effect QTL affecting stay-green in sorghum are localized on SBI02 (*Stg3*), SBI03 (*Stg1, 2*), and SBI05 (*Stg4*) as well as on other chromosomes (Tao et al., 2000), prior QTL analysis in some cases was based on genetic maps with a limited number of molecular markers (Crasta et al., 1999; Xu et al., 2000).

In this study QTL that affect expression of stay-green and leaf dhurrin content were mapped in a RIL population derived from BTx642 (high dhurrin, source of stay-green) and Tx7000 (low dhurrin, non stay-green) that had been used previously for mapping stay-green QTL (Xu et al., 2000). The analysis revealed a main effect QTL for dhurrin content on SBI01 that aligned with the cluster of genes involved in dhurrin biosynthesis, and a previously unreported QTL for stay-green.

Materials and Methods

Plant Material

A recombinant inbred (RI) mapping population consisting of 90 F_{12} lines derived from the parents BTx642 and Tx7000 was used for genetic mapping in this study. The RIL parents, population, and near isogenic lines have been studied extensively for QTL and casual physiological mechanisms associated with the stay-green phenotype in sorghum (Borrell et al., 2014; Harris, 2007; Xu et al., 2000). BTx642 (B35) is a BC_1 selection from BTx406, a 4-dwarf line used for conversion of tall photoperiod sensitive sorghum into short, early flowering genotypes, and IS12555, an excellent source for stay-green (Borrell et al., 2000). Tx7000 (Caprock) is a historically important restorer

line in the United States that is pre-flowering drought tolerant, but is a poor stay-green line.

Dhurrin Phenotyping

The BTx642/Tx7000 RIL population was grown and screened for leaf dhurrin concentration in four environments in 2013: Puerto Rico, College Station, Lubbock-USDA, and Lubbock-Texas A&M AgriLife. In all locations, a randomized complete block design with two replications was used. Sorghum seeds were planted at a rate of 70 seeds per plot at a depth of 4 cm. A plot in each environment was defined as a single row 5.2 meters long. Row spacing in each environment was 1.0 m except for the College Station location, which was 0.76 m. Agronomic practices considered standard for sorghum were used in each environment. All environments received supplemental irrigation as needed to minimize visual drought stress. Each environment also received a pre-plant fertilizer application of varying quantity with exception of the Lubbock-AgriLife location.

Dhurrin was extracted from the leaves of each RIL approximately at anthesis in each environment. Five leaf punches measuring 1.0 cm in diameter were taken from five representative plants within each RIL plot for a total of five pooled samples per entry. Punches were taken on the youngest, fully exposed leaf, excluding the flag leaf. The leaf punches were taken midway between the blade tip and base and did not include the midrib. The leaf punches were immediately placed on ice in the field and immediately returned to the laboratory. Dhurrin from the five leaf punches was extracted in 1 mL 80% ethanol at 60°C for one hour followed by five minutes at room

temperature. The extract was centrifuged for 10 min at 10,000 RPM and the resulting supernatant was transferred to an Eppendorf tube and dried with a vacuum centrifugation system (Savant Instruments Inc.) set on low drying. The extract was suspended in 200 µl of deionized water and separated using a YMC polyamine II column with a mobile phase of 75% acetonitrile (C_2H_3N) at a flow rate of 1.5 mL/min. A standard curve was used to quantify the concentration of leaf dhurrin expressed as $\mu g/cm^2$.

Stay-Green Phenotyping

The parents and 90 RILs were evaluated under post-flowering drought stress conditions in Corpus Christi, Lubbock, and Halfway Texas in 1993, 1994, 1997, and 1998. Plots were defined as 4.9 m long and 1.0 m wide. Stay-green data from the environments described above was used for this study because they represent the environments used to originally map stay-green QTL in sorghum and because the 2013 environments used for dhurrin analysis did not produce post-flowering drought stress where definitive stay-green phenotypes could be observed.

The experimental design was a randomized complete block design with three replications per entry. All irrigation was terminated at anthesis which allowed post-anthesis drought stress to occur later in the growing season. Average high temperatures during the growing season for each year were around 33°C with low relative humidity; conditions that are typical of West Texas, the western High Plains, and many grain sorghum producing regions around the world.

Stay-green was visually scored on the parents, and RIL population on a plot basis on a scale of 1 to 5 based on the degree of visual plant greenness or senescence at

physiological maturity. A rating of 1 indicated completely green leaves with no senescence. A rating of 5 corresponded to no visual greenness in the leaves with complete plant death.

Genetic Map

The genetic map of BTx642/Tx7000 was provided by Dr. John Mullet (Texas A&M University). The RIL population was genotyped using a digital genotyping (DG) method previously described by Morishige et al. (2013). Briefly, plant tissue from each RIL and parent were grown and genomic DNA was isolated using the FastPrep protocol. The DNA was digested with the six-base restriction enzyme *Ngo*MIV (New England BioLabs). The DNA template was prepared, sequenced, and analyzed as previously described (Morishige et al., 2013; Truong et al., 2014). A total of 897 unique DG markers spanning the ten sorghum chromosomes were used for genetic map construction and QTL analysis.

Data analysis and QTL Mapping

Statistical analysis of phenotype data was performed using JMP® software version 10.0 (SAS Institute). Leaf dhurrin and stay-green data were transformed using the square root function to improve homogeneity of error variances. Multivariate analysis was performed using the Pearson method. Coefficient of variation (CV) was calculated according to Bernardo (2010). The transformed mean dhurrin and stay-green rating from each environment were used for composite interval mapping (CIM) QTL analysis with 1000 permutations set at a confidence level of 0.05 in QTL Cartographer® version 2.5. A walk speed of 1.0 cM was used and only QTL above the significant LOD

threshold are reported. The QTL size was reported as two LOD units from the QTL peak and R^2 was reported to determine the percent variation explained (PVE). The additive QTL effect is relative to the Tx7000 allele.

Results and Discussion

Five QTL that modify dhurrin content per unit leaf area were identified using data from the BTx642/Tx7000 RIL population (Table 13). A QTL located on SBI01 (LOD 10-15) with a peak located between 2.5-2.9 cM explained 29-48% of the variance observed. The genomic region from 2.3-2.9 cM spans 1.13 to 1.17 Mbp of SBI01 and includes two of the three genes involved in dhurrin biosynthesis (CYP79A1, UGT85B1). Alleles from BTx642 in this locus were associated with higher leaf dhurrin accumulation. Dhurrin QTL located on SBI 03, 04, 06 and 08 were detected in a subset of the environments (Table 13). These QTL had lower LOD scores and accounted for less of the phenotypic variation in dhurrin levels (Table 13). Two QTL located on SBI08 at 49.7cM and 57.6 cM were identified in the low N TAES13 environment.

Table 13. Dhurrin QTL identified using *Ngo*MIV DG markers using BTx642/Tx7000 RILs grown in four environments in 2013.

Environment	LG	Peak (cM)	LOD	LOD-1 (cM)	Additive†	R^2	QTL Name
PR13	1	2.5	14.8	1.9	-15.5	0.48	*Dhu1*
PR13	3	57.1	2.7	5.8	-6	0.06	*unknown*
CS13	1	2.9	13.3	2.1	-16.9	0.29	*Dhu1*
CS13	6	48.3	4.1	3.6	8.3	0.07	*unknown*
USDA13	1	2.9	10.7	3.1	-8.4	0.29	*Dhu1*
USDA13	3	54.1	3.7	7.5	-4.7	0.08	*unknown*
USDA13	6	102.9	3.1	4.9	4.1	0.07	*unknown*
TAES13	1	2.9	13.8	2.6	-12.7	0.36	*Dhu1*
TAES13	4	144.9	3.6	11.1	5.6	0.07	*unknown*
TAES13	8	49.7	8.5	5.2	-9.4	0.2	*unknown*
TAES13	8	57.6	6.5	2.3	-8.7	0.17	*unknown*

† Relative effect of RTx7000 allele

Four QTL, *Stg1-4*, that modify expression of stay-green were previously located on chromosomes 2 (*Stg3*), 3 (*Stg1,2*) and 5 (*Stg4*) using phenotypic data on relative rates of leaf senescence under water limiting conditions using the BTx642/Tx7000 RIL population (Xu et al., 2000b; Subudhi et al., 2000). Re-analysis of stay-green QTL using the original data from Xu et al. (2000b) and the DG-marker based genetic map re-confirmed the *Stg1-4* QTL in one or more of the environments analyzed. The *Stg1-4* QTL each account for 7-14% of the stay-green phenotypic variation (Xu et al., 2000b). In addition, a stay-green QTL, which was not identified in the previous studies, was

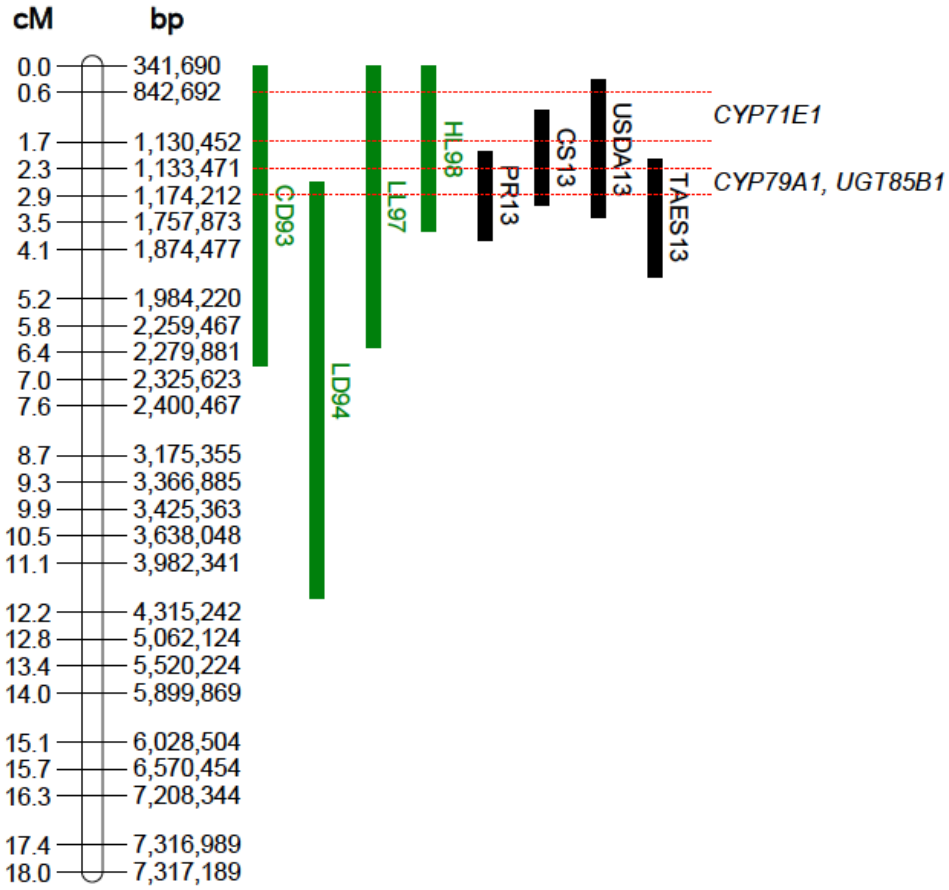
identified on SBI01 (*Stg5*) in all four environments with a peak located between 2.4-2.9 cM (Table 14) (Figure 3). This QTL explained 8-14% of the phenotypic variation depending on environment. The lack of DNA markers spanning this region of SBI01 in the genetic map used by Xu et al. (2000) is the most likely reason this QTL was not found in the original analysis.

Table 14. Stay-green QTL identified using *Ngo*MIV DG markers using BTx642/Tx7000 RILs grown in four environments in 2013.

Environment	LG	Peak (cM)	LOD	LOD-1 (cM)	Additive†	R^2	QTL Name
CD93	1	4.1	4.5	6.6	0.27	0.12	*Stg5*
CD93	3	74.6	4.9	3.4	0.27	0.06	*Stg2*
CD93	3	80.8	5.2	5.1	0.23	0.07	*Stg1*
CD93	5	56.9	4.1	9.1	0.25	0.11	*Stg4*
LD94	1	7.7	4.6	7.7	0.23	0.11	*Stg5*
LD94	2	97.4	3.7	8.7	0.27	0.13	*Stg3*
LD94	3	73.5	4.7	2.4	0.26	0.12	*Stg2*
LD94	3	108.4	5.3	2	0.26	0.14	*Stg1*
LL97	1	2.9	2.8	6.4	0.18	0.08	*Stg5*
LL97	3	49.9	6.1	4.8	0.29	0.19	*unknown*
LL97	10	91.1	3.1	4.7	-0.18	0.09	*unknown*
HL98	1	0	4.1	3.6	0.11	0.14	*Stg5*
HL98	3	93.5	2.9	4.4	0.1	0.11	*Stg1*

† Relative effect of RTx7000 allele

Figure 3. Co-alignment of stay-green (*Stg5*) QTL with dhurrin (*Dhu1*) QTL on SBI01. Black bars represent dhurrin QTL identified in 2013. Green bars represent a new stay-green QTL identified in the present study using phenotypic data from four different environments collected by Xu et al. (2000b) along with the higher density genetic map constructed using DG technology (Weers, 2011). The red dashes and corresponding gene ID's represent known dhurrin biosynthetic genes.



The sequence variants in the three genes encoding enzymes involved in dhurrin biosynthesis present in Tx7000 are also found in Blackhull kafir, a line imported into the U.S. from southern Africa late in the 19th century (Smith and Frederiksen, 2000). Blackhull kafir was commonly used in U.S. grain sorghum breeding programs between 1900-1930 and is in the pedigree of Tx3042, BTx399, BTx378, Tx7078, and Tx7000. Blackhull kafir or lines derived therefrom, were also used in breeding BTx623, BQL41 and BTx406 which have all been used as seed parents for hybrid grain sorghum breeding. All of these genotypes have low leaf dhurrin levels and were the genetic base for the majority of grain sorghum breeding lines in the U.S. The presence of the low dhurrin haplotypes across the cluster of genes involved in dhurrin biosynthesis in Blackhull kafir indicates that selection, natural or human, of low dhurrin lines for grain sorghum production most likely occurred prior to importation of this genetic material from Africa in 1890. Subsequent use and selection for genotypes with higher grain yields resulted in selection of lines with lower dhurrin suggests that lower levels of leaf dhurrin could contribute directly or indirectly to higher grain yield. Hayes et al. (2015) identified variability for average leaf dhurrin content between the major races of sorghum and identified the kafir sorghums as primarily low dhurrin.

Because sorghum is also used for forage, selection against high levels of dhurrin in these materials may also have also contributed to predominantly lower dhurrin breeding lines in sorghum breeding programs. Alleles contributing to low dhurrin level in BTx406 help explain results from a recent association study based on analysis of dhurrin levels in lines derived from the sorghum conversion program (Hayes et al.,

2015). The same region of SBI01 identified here was associated with variation in dhurrin level along with a region on SBI08 that aligned with genes encoding dhurrinase (Hayes et al., 2015). BTx406, 4-dwarf kafir sorghum, was used extensively for converting exotic accessions to short, early flowering genotypes (Klein et al., 2008). The fact that BTx406 also contains an allele for low dhurrin, whereas many exotic sorghum genotypes have high dhurrin, may help explain the association results (Hayes et al., 2015). Hayes et al. (2015) showed that there were many genotypes with leaf dhurrin levels >100 μg/cm^2 (i.e., SC144, SC728). Leaf dhurrin was also measured for BTx642 within the same study and contained approximately half the dhurrin (~63 μg/cm^2) of the highest converted lines listed above (Hayes, unpublished results). In addition, there were converted lines with leaf dhurrin levels <10 μg/cm^2, much lower than Tx7000 (~21μg/cm^2), indicating the genetic basis of variation in leaf dhurrin levels in sorghum is complex and much more variable than dhurrin levels observed in most high yielding grain sorghum lines used in public and private breeding programs in the U.S. (Hayes, unpublished results). The identification of small affect dhurrin QTL in the BTx642/Tx7000 RIL population on SBI03, SBI04, SBI06, SBI08 and SBI10 indicates numerous genes, alleles, and pathways affect leaf dhurrin levels in sorghum.

Conclusion

A QTL (*Dhu1*) that affects leaf dhurrin level was identified on SBI01 using a RIL population derived from BTx642 and Tx7000. The QTL accounted for 29-48% of the phenotypic variance in dhurrin concentration per unit leaf area in leaves near the top

of the canopy at anthesis in four environments. Leaf dhurrin levels at anthesis were approximately 2.5-times higher in BTx642, a genotype known as an excellent source of the stay-green drought tolerance trait, than in Tx7000 (Rosenow and Clark, 1995). The QTL that associates with dhurrin level was located on SBI01 in a region that encodes three genes involved in dhurrin biosynthesis, CYP79A1, CYP71A1, and UGT85B1, where the dhurrin QTL peak aligned with CYP71A1 and UGT85B1.

Burke et al. (2013) identified a correlation between genotypes with elevated leaf dhurrin level and genotypes with higher stay-green leaf and plant death (LPD) rating following exposure to post-anthesis water limitation. BTx642 and lines derived therefrom had relatively high levels of leaf dhurrin and stay-green ratings. BQL41, an Australian genotype with an intermediate stay-green rating, contained intermediate dhurrin levels, and Tx7000 had low stay-green ratings and accumulated low levels of leaf dhurrin at anthesis (Burke et al., 2013; Rosenow and Clark, 1995). Analysis of the genetic basis of stay-green in BTx642 (B35) (Borrell et al., 2014; Crasta et al., 1999; Harris et al., 2007; Tuinstra et al., 1997; Xu et al., 2000), SC56 (Kebede et al., 2002), BQL41 (Tao et al., 2000) and E36-1 (Haussman et al., 2002) identified >10 QTL that affect expression of stay-green (Mace and Jordan, 2011). Four stay-green QTL with relatively large effects, *Stg1-Stg4*, were identified through analysis of the RIL population derived from BTx642 and Tx7000 (Xu et al., 2000; Subudi et al., 2000). The current study based on the same BTx642/Tx7000 RIL population identified an additional stay-green QTL, designated *Stg5*, on SBI01 that is highly heritable and accounts for ~8-14% of the phenotypic variation for stay-green in four different environments where plants

were exposed to water deficit post-anthesis. A small effect QTL for retention of green leaf area was located at the end of SBI01 in one prior study (Haussmann et al., 2002). This stay-green QTL, corresponding to alleles derived from the stay-green source E36-1, could be related to the stay-green QTL identified on SBI01 in the current study.

The physiological mechanisms of *Stg1-4* has been analyzed using near-isogenic lines (Borrell et al., 2014). These studies showed that to varying degrees, individual *Stg*-loci decreased tiller number, leaf size, and crop water use prior to anthesis, thereby making more water available post anthesis for grain production (Borrell et al., 2014a). Additional stay-green loci are co-localized with QTL for root angle (Mace et al, 2012). The QTL for leaf dhurrin level and stay-green located on SBI01 does not overlap with *Stg1-4*, or QTL for root angle, consistent with the hypothesis that variation in leaf dhurrin level is affecting the rate of leaf senescence post-anthesis under water limiting conditions (Burke et al., 2013). Previous research established that variation in rates of leaf senescence post-anthesis are related to the dynamics of N-demand by the panicle (grain) and N-supply from continued N-uptake by roots and N-mobilization from stems and leaves (Borrell et al., 2000; Borrell et al., 2001). Water deficit post-anthesis reduces N-uptake by the root system accelerating N-mobilization from stems and leaves leading to earlier leaf senescence. Increased levels of dhurrin could delay leaf senescence in many possible ways. Dhurrin could be providing a source of N for grain filling under water limiting conditions thereby delaying the onset of leaf senescence. The levels of leaf dhurrin represent ~1-5% of total leaf N at anthesis and remobilization could delay leaf senescence to a small extent. Remobilization of cyanogenic glucosides has been

well documented in several species therefore the dynamics of dhurrin mobilization during grain filling deserves further research (Gleadow and Møller, 2014). Another hypothesis is that dhurrin synthesis alters leaf growth and development by affecting N signaling in the developing leaf. Mutations in CYP79A1, the first committed step in the pathway, resulted in acyanogenic plants that grew slower than the wild type (Blomstedt et al., 2012). Interestingly, sorghum TILLING lines that accumulated low levels of leaf dhurrin, but without mutations in genes involved in dhurrin biosynthesis, were leafier and had more tillers than controls (Blomstedt et al., 2012). This suggests that synthesis of elevated levels of leaf dhurrin could cause a reduction in leaf area, a key trait associated with the function of *Stg1-4*. Additionally, high dhurrin TILLING lines derived from BTx623 have been observed to have reduced vigor in the field and produced narrow and erect leaves with reduced total leaf area (Hayes, unpublished results).

Genotypes such as BTx623, BTx378, RTx7000, and RTx436 have been selected because they are useful in grain production and contribute to high yielding grain hybrids (Rooney, personal communication). These genotypes have lower levels of leaf dhurrin at anthesis compared to BTx642, a line with relatively low grain yield. The correlation between low leaf dhurrin and high grain yield deserves further analysis because if high dhurrin reduces grain yield, especially in favorable environments with no drought stress, then selection for stay-green QTL that contribute to higher grain yield in water limiting conditions should avoid selection for the QTL for high dhurrin and focus instead on selecting for beneficial alleles associated with *Stg1-4* and *Stg* QTL aligned with optimal

root angle for each region of production. The potential negative association between elevated dhurrin levels and grain yield could be a consequence of altered N-availability via dhurrin sequestration or signaling. The sequestration of available N in the form of dhurrin could affect plant responses to water deficit pre-flowering and post-flowering, their potential for grain yield production, as well as resistance to pests and disease.

CHAPTER V

EVALUATION OF A DIVERSE SET OF BREEDING LINES FOR DHURRIN CONTENT AND STAY-GREEN

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is C_4 cereal grass species that has many uses as a food, feed, forage, and feedstock (Rooney, 2007). Sorghum is traditionally cultivated on marginal lands around the world and routinely is affected by drought stress. Sorghum is commonly known to be drought tolerant, especially compared to other cereal grains line rice and maize.

Stay-green in sorghum is an economically important drought tolerance trait in some production regions where drought post-anthesis is common (Jordan et al, 2012). Stay-green plants maintain green leaf area longer under drought post-anthesis, remain photosynthetically active longer, and produce more grain yield under drought conditions (Borrell et al., 2000b; Jordan et al., 2012).

Dhurrin is a cyanogenic glucoside produced by *Sorghum bicolor* and other sorghum species (Rhykerd and Johnson, 2007). Dhurrin is known to be non-volatile compound in isolation, but physical disruption of plant tissue by animal herbivory or drought stress, allow for the production of hydrogen cyanide (HCN) produced by the interaction of dhurrin and catabolic dhurrinase enzymes (Busk and Moller, 2002). Dhurrin has also been proposed to be an available source of N with osmoprotectant properties (Burke et al., 2015). Burke et al. (2013) recently identified multiple stay-green breeding lines that contain elevated leaf dhurrin levels. LPD ratings from previous

environments were used to associate elevated leaf dhurrin levels with stay-green. Commonly used stay-green germplasm like BTx642, B4R, and SC1154-14E, contained 3-4x higher dhurrin levels compared to senescent sorghums (Burke et al., 2013).

Although a strong association between stay-green and leaf dhurrin was observed by Burke et al. (2013), additional research is needed to evaluate stay-green (LPD) ratings and leaf dhurrin all within the same environment. The objectives of this study are to analyze genotype, environment, and GxE effects for leaf dhurrin, leaf sugars, and stay-green response for ten diverse grain sorghum breeding lines. Additionally, correlations between traits are assessed and trait repeatability is estimated on an entry-mean basis.

Materials and Methods

Plant Material and Experimental Design

A set of ten diverse breeding lines varying in stay-green response were used in this study (Table 15). The breeding lines vary for adaptation, maturity and stay-green response. The stay-green lines also vary for the genetic source of stay-green.

Table 15. List of ten diverse breeding lines evaluated for dhurrin, leaf sugars, and stay-green in 2014.

Line	Pedigree	Class	Source
BTx642	BTx406/IS12555	Stay-green	Xu et al., 2000
R9188	SC599-6sel	Stay-green	Rosenow et al., 1983
1790E	SC56/SC33	Stay-green	Rosenow et al., 1983
B4R	BTx406/Rio	Stay-green	Burke et al., 2013
B1778	SC56/SC33	Stay-green	Rosenow et al., 1983
Tx7000	Caprock, Kafir-Milo	Senescent	Xu et al., 2000
RTx437	SC120sel/RTx430	Senescent	Burke et al., 2013
BTx623	BTx3197/SC170-6-4	Senescent	Rosenow et al., 1983
BTx3042	Redbine,Kafir-Milo	Senescent	Burke et al., 2013
BTx378	Redlan, Kafir	Senescent	Tenkouano et al., 1993

The lines were grown in replicated field trials in four locations in 2014. These environments were designated as 14WE, 14CA, 14CW 14LB. Weslaco (WE) is located in the southern tip of Texas and is a humid, but arid sub-tropical environment. Corpus Christi (CA, CW) is also a humid but semi-arid environment located along the Texas gulf coast. Lubbock (LB) is located on the High Plains of Texas and has a dry, temperate climate. All locations are established sorghum production regions of Texas and commonly experience post-flowering drought stress. In all locations, a randomized complete block design (RCBD) with three replications was used. Sorghum seeds were planted at a rate of 70 seeds per plot at a depth of 4 cm. A plot in each environment was defined as a single row 5.2 meters long. Row spacing in each environment was 1.0 m

wide. The Weslaco and Lubbock environments were furrow irrigated as needed to minimize visual drought stress until flowering. The Corpus Christi environments were rain fed only and did not receive supplemental irrigation. All seed were treated with a seed treatment mixture of Captan®, Concept® and Gaucho® to allow for the application of Dual herbicide for pre-germination weed control.

Stay-Green Phenotyping

Stay-green ratings in the form of leaf and plant death rating (LPD) were scored in each environment on a plot basis on a scale of 1 to 5 based on the degree of visual plant greenness or senescence at physiological maturity as per Xu et al. (2000b). A rating of 1 indicated a completely green plant with green leaves and no senescence. A rating of 5 corresponded to no visual greenness in the leaves accompanied with complete plant death.

Leaf Dhurrin and Sugar Analysis

Leaf dhurrin, glucose, sucrose, and fructose were extracted from the lines approximately at mid-anthesis in each location. Five total leaf punches from each entry measuring 1.0 cm in diameter were taken from five random but representative plants within each RIL plot. Punches were taken on the youngest, fully exposed leaf, excluding the flag leaf. The leaf punches were taken midway between the blade tip and base and did not include the midrib. The leaf punches were immediately placed on ice in the field and were quickly returned to the laboratory. All HPLC analysis was performed at the Cropping Systems Research Laboratory in Lubbock, TX. Dhurrin and sugars from the five leaf punches was extracted in 1 mL 80% ethanol at 60°C for one hour followed by

five minutes at room temperature. The extract was centrifuged for 10 minutes at 10,000 RPM and the resulting supernatant was transferred to an Eppendorf tube and dried with a vacuum centrifugation system (Savant Instruments Inc.) set on low drying. The extract was suspended in 200 µl of deionized water and separated on a YMC polyamine II column with a mobile phase of 75% acetonitrile (C_2H_3N) at a flow rate of 1.5 mL/min. A standard curve was used to quantify the concentration of dhurrin and sugars expressed as $\mu g/cm^2$.

Statistical Analysis

Individual data from each location were analyzed to determine if variances were homogeneous. Heterogeneity of error variances was not detected so a combined analysis was performed. The statistical model used for the combined analysis was:

$$Dependent\ Variable = \mu + Genotype + Environment + Rep[Env] + Error.$$

JMP® Version 10.0.0 was used for all statistical analysis. Genotype was considered a fixed effect and all other sources of variation were considered random effects. Raw HPLC data was log transformed to correct for ANOVA normality assumptions. Multivariate analysis was performed using the Pearson method. Coefficient of variation (C.V.) was calculated according to Bernardo (2010). Repeatability of traits was calculated similarly to a heritability estimate:

$$Repeatability = \sigma^2_{G} / [\sigma^2_{G} + (\sigma^2_{GxE/E}) + (\sigma^2_{error/ER})]$$

Results and Discussion

In the combined analysis, genotype effects were significant for all traits measured (Table 16). The environment effect was also significant for all traits except for stay-green (LPD). The interaction of genotype and environment (GxE) was significant for dhurrin and fructose, although the magnitude of the interaction was minimal (Table 16). Repeatability for all traits was high, ranging from 0.87 (fructose) to 0.95 (glucose).

Table 16. Mean squares for leaf composition traits and LPD from combined data from the stay-green vs. non stay-green test in four environments in 2014.

Source of Variation	df	Dhurrin†	Glucose†	Fructose†	Sucrose†	LPD‡
Environment (E)	3	2.12***	4.03***	8.75***	15.47***	0.25
Rep[E]	8	0.08**	0.32***	2.76***	0.89***	0.49*
Genotype (G)	9	3.23***	0.48***	2.82***	0.51***	9.0***
GxE	27	0.13***	0.07	1.7**	0.13	0.32
Error	72	0.04	0.05	0.04	0.08	0.21
R^2		0.93	0.85	0.86	0.92	0.86
CV (%)		5.0	9.6	5.0	7.5	15.3
Repeatability		0.89	0.95	0.87	0.92	0.92

*,**,*** Significant at 0.10, 0.05, and 0.01, respectively

† $\mu g/cm^2$ of leaf tissue

‡ Leaf and plant death rating where 1=green and 5=fully senesced

Weslaco (WE) produced the highest dhurrin levels with an average of 50.0 $\mu g/cm^2$ and Corpus Annex (CA) produced the lowest dhurrin levels at 29.2 $\mu g/cm^2$. Environmental differences for leaf dhurrin content did not receive further investigation because N fertilizer, known to greatly affect dhurrin accumulation, was not consistent across environments.

Dhurrin was strongly correlated (-0.62) with stay-green in this study (Table 17). Associations between leaf dhurrin and stay-green have been observed previously, thus, the strong correlation observed in this study between dhurrin and visual stay-green is confirmation of previous results (Burke et al., 2013; Hayes et al., 2015b; McBee and Miller, 1980). Additionally, stay-green was modestly (-0.14) correlated with leaf sucrose concentrations (Table 17). Previous research has identified that two known stay-green lines (BTx642, 1790E) contained high leaf sucrose concentrations at anthesis than did two known senescent lines (Tx7000, BTx623) (Burke et al., 2010). Burke et al. (2010) also identified that quantum efficiency (Fv/Fm), a predictive bioassay used in identifying stay-green breeding lines, significantly correlated (0.67) with leaf sucrose levels.

Table 17. Correlations of dhurrin, leaf sugars, and stay-green (LPD) in four environments in 2014.

Trait	Dhurrin†	Fructose†	Glucose†	Sucrose†	LPD‡
Dhurrin	-				
Fructose	-0.11	-			
Glucose	**-0.16***	**0.97*****	-		
Sucrose	0.05	0.09	0.04	-	
LPD‡	**-0.62****	-0.08	-0.08	**-0.14***	-

*, **, *** Significant at 0.10, 0.05 and 0.01, respectively
† $\mu g/cm^2$ of leaf tissue
‡ Leaf and plant death rating where 1=green and 5=fully senesced

The breeding lines varied greatly for dhurrin in this study (Table 18). Combined across four environments, leaf dhurrin ranged from 84.8 μg/cm^2 (B1778) to 20.0 μg/cm^2 (Tx7000) and generally separated into two distinct classes based on the presence/absence of stay-green (Table 18). BTx642, a standard for stay-green, averaged 60.0 μg/cm^2 and was statistically higher than all senescent lines. Even though it was numerically higher in dhurrin, the breeding line R9188 was the only stay-green line that did not have statistically higher dhurrin levels than senescent lines (Table 18).

Table 18. Averages for leaf composition traits and LPD of 10 breeding lines varying for stay-green evaluated across four environments in 2014.

Pedigree	Class	Dhurrin†	Glucose†	Fructose†	Sucrose†	LPD‡
B1778	Stay-green	84.8	73.4	57.6	64.5	2.5
1790E	Stay-green	66.7	46.5	40.0	42.5	2.3
BTx642	Stay-green	60.0	82.1	70.4	78.4	1.5
B4R	Stay-green	51.7	80.6	60.4	78.9	2.3
R9188	Stay-green	45.9	95.8	63.8	65.8	2.4
RTx437	Senescent	28.3	80.3	64.9	44.9	3.7
BTx378	Senescent	23.5	75.1	59.5	39.2	3.8
BTx623	Senescent	23.1	57.8	47.5	63.9	3.8
BTx3042	Senescent	23.0	71.2	55.3	54.5	3.6
Tx7000	Senescent	20.9	90.2	67.5	67.3	3.8
$LSD_{p<.05}$		9.8	27.1	19.6	39.8	0.5
CV (%)		5.0	10.4	9.5	19.9	17.1

† μg/cm^2 of leaf tissue

‡ Leaf and plant death rating where 1=green and 5=fully senesced

The relative ranks of breeding lines for dhurrin concentration differ between environments but the magnitude is minimal (Table 19). Among the stay-green lines, R9188 had the lowest dhurrin concentrations in three of the four environments in this

study (Table 18). In the WE environment, R9188 had the second highest dhurrin levels where B1778 being the highest. This shift in the concentrations in R9188 may account for the significant GxE effect.

The breeding lines were also re-confirmed as stay-green or non-stay-green based on visual LPD rating (Table 18 and Table 20). BTx642 had the best LPD rating within each environment (Table 20). Rank differences for LPD within different environments was observed, but the differences were minimal and the combined analysis GxE effect was non-significant (Table 20).

Table 19. Least significant differences for leaf dhurrin for 10 different grain sorghum lines varying for the stay-green phenotype in Corpus Christi annex (CA), Corpus Christi West (CW), Lubbock (LB) and Weslaco (WE) in 2014. Color code green classified as stay-green genotype and yellow color classified as senescent genotype.

Leaf Dhurrin (ug/cm^2)			
CA		CW	
Genotype	LS Mean	Genotype	LS Mean
1790E	70.4	B1778	91.6
B1778	51.0	BTx642	66.9
BTx642	38.5	1790E	60.5
B4R	27.5	B4R	52.8
R9188	24.8	R9188	33.0
BTx378	17.7	RTx437	29.2
BTx623	16.8	BTx623	24.2
Tx7000	15.3	BTx378	22.4
BTx3042	14.9	BTx3042	20.3
RTx437	14.6	Tx7000	19.8
$LSD_{p<.05}$	10.8	$LSD_{p<.05}$	19.6

LB		WE	
Genotype	LS Mean	Genotype	LS Mean
B1778	90.08	B1778	106.35
BTx642	78.04	R9188	82.79
1790E	73.81	B4R	79.61
B4R	46.67	1790E	61.63
R9188	42.84	BTx642	56.36
RTx437	41.15	RTx437	28.10
BTx3042	30.54	BTx3042	26.21
BTx378	30.00	BTx378	23.72
BTx623	29.54	Tx7000	22.92
Tx7000	25.22	BTx623	21.95
$LSD_{p<.05}$	17.7	$LSD_{p<.05}$	23.0

Table 20. Least significant differences for leaf and plant death rating (LPD) for 10 different grain sorghum lines varying for the stay-green phenotype in Corpus Christi annex (CA), Corpus Christi West (CW), Lubbock (LB) and Weslaco (WE) in 2014. Color code green classified as stay-green genotype and yellow color classified as senescent genotype.

Leaf and Plant Death Rating (LPD)			
CA		CW	
Genotype	LS Mean	Genotype	LS Mean
BTx623	4.0	BTx378	4.2
BTx378	3.8	BTx623	4.2
Tx7000	3.7	Tx7000	4.0
RTx437	3.7	BTx3042	3.7
BTx3042	3.2	RTx437	3.7
B1778	3.0	B1778	2.7
B4R	2.3	R9188	2.3
R9188	2.0	B4R	2.3
1790E	1.8	1790E	2.0
BTx642	1.3	BTx642	1.7
$LSD_{p<.05}$	1.1	$LSD_{p<.05}$	1.0

LB		WE	
Genotype	LS Mean	Genotype	LS Mean
BTx3042	4.2	RTx437	4.2
Tx7000	4.0	BTx378	3.8
BTx623	3.8	BTx3042	3.7
RTx437	3.7	Tx7000	3.7
BTx378	3.5	BTx623	3.3
1790E	2.7	1790E	2.8
B4R	2.7	R9188	2.5
R9188	2.7	B1778	2.2
B1778	2.3	B4R	2.0
BTx642	1.3	BTx642	1.8
$LSD_{p<.05}$	0.9	$LSD_{p<.05}$	1.1

Leaf sucrose also varied greatly in this study (Table 17). B4R contained the highest concentrations of sucrose (78.9 ug/cm^2), and BTx378 contained the lowest concentration of sucrose (38.2 ug/cm^2). B4R is a derivative of Rio, a sweet sorghum selected for high sugar (Brix) content in the stems. BTx642 and R9188, two known stay-green lines, also consistently contained higher sucrose concentrations in all environments (Table 21). The line 1790E, a known stay-green line, produced relatively low leaf sucrose concentrations in all environments (Table 21). Previous studies have associated high leaf sucrose concentrations with stay-green using the breeding lines BTx642 and R9188. Results from this study indicate that some stay-green lines do indeed contain elevated leaf sugars, but other known stay-green lines (1790E) contain relatively low leaf sucrose levels, indicating that leaf sugar content is at best a contributory factor in stay-green but it is not a primary factor.

Table 21. Least significant differences for leaf sucrose for 10 different grain sorghum lines varying for the stay-green phenotype in Corpus Christi annex (CA), Corpus Christi West (CW), Lubbock (LB) and Weslaco (WE) in 2014. Color code green classified as stay-green genotype and yellow color classified as senescent genotype.

Leaf Sucrose (ug/cm^2)			
CA		CW	
Genotype	LS Mean	Genotype	LS Mean
R9188	89.4	BTx642	34.4
BTx642	83.6	B4R	33.0
B4R	69.4	R9188	29.8
Tx7000	58.4	RTx437	28.6
BTx623	53.4	BTx3042	26.8
BTx3042	51.8	Tx7000	26.8
B1778	42.7	BTx378	25.5
BTx378	41.9	B1778	25.5
RTx437	37.7	BTx623	25.2
1790E	32.8	1790E	23.9
$LSD_{p<.05}$	51.9	$LSD_{p<.05}$	7.7

LB		WE	
Genotype	LS Mean	Genotype	LS Mean
BTx642	48.6	B4R	171.0
B4R	42.1	B1778	169.6
BTx623	40.7	Tx7000	145.9
Tx7000	23.7	BTx623	136.6
BTx3042	23.2	BTx642	132.5
B1778	20.3	BTx3042	116.4
R9188	20.2	R9188	108.5
BTx378	18.7	RTx437	100.7
1790E	17.5	1790E	95.9
RTx437	12.8	BTx378	66.3
$LSD_{p<.05}$	31.8	$LSD_{p<.05}$	45.2

Conclusion

Ten diverse grain sorghum lines were evaluated in four environments in 2014 for leaf dhurrin, sugar concentration and visual stay-green ratings. The lines selected are a diverse set of grain breeding lines that represent the phenotypic extremes of stay-green and non-stay-green grain sorghum and are lines commonly used in breeding programs selecting for pre and post-flowering drought tolerance. The lines were selected exclusively based on published stay-green ratings and were not pre-selected based on leaf dhurrin content.

As expected, the lines separated into two distinct groups based on stay-green and they also differentiated in similar groups for leaf dhurrin concentration. Stay-green lines consistently contained 2-3x the leaf dhurrin content at anthesis than did non-stay-green breeding lines. Leaf dhurrin also varied greatly within this study. Combined across all environments, B1778 contained the highest concentrations of dhurrin (84.8 $\mu g/cm^2$) and Tx7000 contained the least (20.9 $\mu g/cm^2$). For every classified stay-green line, leaf dhurrin levels at anthesis were at least 2x the levels observed in non-stay-green breeding lines.

Leaf sucrose was also correlated with LPD rating in this experiment. B4R contained the highest leaf sucrose, and BTx378 contained the lowest. Although leaf sucrose was correlated with LPD rating the trend was not exclusive. Specifically, genotype 1790E, a known stay-green, line produced low leaf sucrose concentrations in all environments.

Repeatability for all traits was very high in this study, indicating that similar results can be observed within similar environments and breeding lines. The very high repeatability observed in this study could be inflated due to the exclusion of environments in 2014 that did not produce profound differences in visual stay-green response. Specifically, three additional locations were not included in the analysis because the stay-green phenotype was not expressed in those specific environments due to rainfall late in the season. 2014 was an excellent year for the evaluation of stay-green in some environments, as evidenced by clear separation within breeding lines and a relatively low CV, but the inclusion of environments with moderate or no differences for LPD would decrease repeatability and made effects due to environment more profound. LPD ratings for stay-green are notoriously difficult to re-produce, so results from this study only represent the ideal evaluation environments of 2014 and the large differences for stay-green response already known within the breeding lines.

Burke et al. (2013) first described a relationship between elevated leaf dhurrin levels at anthesis and stay-green. This study expands on previous work by Burke et al. (2013) by measuring stay-green (LPD), leaf dhurrin and leaf sugars within the same environments. The casual effect of high leaf dhurrin at anthesis producing a stay-green response in sorghum is still not fully understood. In chapter IV, a novel stay-green QTL *(Stg5)* was discovered on SBI01 that directly aligns with a dhurrin QTL (*Dhu1*), and the biosynthetic genes for dhurrin. The source of stay-green and dhurrin in that study was BTx642. Results from this study indicate that associations between dhurrin and stay-

green are present beyond the BTx642 genetic background, and that further investigation is needed to fully understand the relationship between high dhurrin and stay-green.

CHAPTER VI

CONCLUSION

Stay-green is an economically important trait in grain sorghum that contributes to increased grain yields in drought that occurs post-anthesis. As drought continues to affect grain sorghum production throughout the world, new screening techniques will be needed to accurately, and quickly identify germplasm that is drought tolerant. Two novel stay-green assays were evaluated in the present study for their efficacy to discriminate between stay-green and non stay-green lines without the need of multiple field studies spanning across many years and locations.

In this study, the quantum efficiency assay (Fv/Fm) did discriminate between the RIL parents BTx642 and Tx7000, but the assay failed to identify differences in stay-green expression within the RILs. This assay is highly sensitive to minor variations in water stress and appears to only work effectively when plants are fully irrigated with no drought stress. Field conditions, especially in environments where periodic drought stress is common and field conditions are not managed extensively, produced erroneous results with high CV values and no statistical separation between RILs. This assay is highly dependent upon stringent protocol adherence, especially in terms of field conditions, leaf tissue collection, and precise temperature treatments during the assay temperature challenge period. These variables appear to also greatly reduce the efficacy of this assay in many environments. The assay was successful in separating BTx642 and Tx7000 under non water-stressed conditions as first described by Burke et al. (2010). This assay is recommended for researchers that are interested in quickly identifying the

phenotypic extremes of stay-green only if the specific growing conditions mandated by the assay can be provided.

Dhurrin was highly effective at discriminating between known stay-green and non-stay-green RIL lines. In all environments evaluated, a high correlation was observed between leaf dhurrin and stay-green. Heritability (h^2) was very high in this study, indicating that breeding progress for leaf dhurrin can be made. BTx642 consistently contained more dhurrin than RTx7000. QTL mapping of leaf dhurrin identified a novel dhurrin QTL on SBI01 that explained a large portion of dhurrin variation in BTx642/Tx7000. QTL analyses of the population using a marker dense DG map with much more marker coverage than the original analysis reconfirmed the stay-green QTL *Stg1-4* in at least a subset of environments. In addition, a new stay-green QTL (*Stg5*) was identified on SBI01. This new QTL is not associated with any of the previously identified stay-green QTL, but is aligned with a novel dhurrin QTL (*Dhu1*) and dhurrin biosynthesis genes.

The association of leaf dhurrin and leaf sucrose to a smaller extent, with stay-green was also observed in a diverse set of ten breeding lines varying for stay-green and genetic background. Importantly, some of the stay-green lines observed contained sources of stay-green that are not derived from BTx642. Both the results obtained from the BTx642/Tx7000 RIL population and the ten diverse breeding lines provide strong evidence that dhurrin biosynthesis is a key trait of stay-green sorghums. Additional studies are needed to identify the relative effect of dhurrin as a stay-green QTL, without the presence of *Stg1-4* QTL. Specifically, the mechanism for exactly how dhurrin keeps

leaves green in sorghum in still unknown. Finally, many physiological and molecular studies are still needed to help identify specifically how dhurrin is involved in the dynamic plant regulatory gene system that affects plant architecture, mobilization of plant resources, and leaf and root growth.

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