Genome-Wide Association Study of Developing Leaves’ Heat Tolerance during Vegetative Growth Stages in a Sorghum Association Panel

Junping Chen*, Ratan Chopra, Chad Hayes, Geoffrey Morris, Sandeep Marla, John Burke, Zhanguo Xin, and Gloria Burow

Abstract
Heat stress reduces grain yield and quality worldwide. Enhancing heat tolerance of crops at all developmental stages is one of the essential strategies required for sustaining agricultural production especially as frequency of temperature extremes escalates in response to climate change. Although heat tolerance mechanisms have been studied extensively in model plant species, little is known about the genetic control underlying heat stress responses of crop plants at the vegetative stage under field conditions. To dissect the genetic basis of heat tolerance in sorghum (Sorghum bicolor (L.) Moench), we performed a genome-wide association study (GWAS) for traits responsive to heat stress at the vegetative stage in an association panel. Natural variation in leaf firing (LF) and leaf blotching (LB) were evaluated separately for 3 yr in experimental fields at three locations where sporadic heat waves occurred throughout the sorghum growing season. We identified nine single-nucleotide polymorphisms (SNPs) that were significantly associated with LF and five SNPs that were associated with LB. Candidate genes near the SNPs were investigated and 14 were directly linked to biological pathways involved in plant stress responses including heat stress response. The findings of this study provide new knowledge on the genetic control of leaf traits responsive to heat stress in sorghum, which could aid in elucidating the genetic and molecular mechanisms of vegetative stage heat tolerance in crops. The results also provide candidate markers for molecular breeding of enhanced heat tolerance in cereal and bioenergy crops.

Core Ideas
• Sorghum could serve as a vital resource of heat tolerance DNA markers.
• Natural variation of leaf traits provides understanding of heat tolerance in sorghum.
• GWAS reveals 14 SNPs with two heat stress responsive traits in sorghum leaves.

Heat stress caused by naturally occurring heat wave events significantly reduces plant growth, agricultural production, and grain quality worldwide, resulting in extensive economic losses (Lobell et al., 2011; Boyer, 1982). The drought and heat stresses that occurred in the 2011 growing season in the Southern Plains and southwest regions of the United States caused more than $5 billion in direct losses to agriculture (NOAA National Centers for Environmental Information, 2017). In 2010 and 2011, heat waves accompanied by drought reduced Russian wheat (Triticum aestivum L.) production by ~30%, causing a shortage in wheat supply and wheat price.

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Abbreviations: BLUP, best linear unbiased predictor value; CSRL, Cropping Systems Research Laboratory; FDR, false discovery rate; GBS, genotype-by-sequencing; GWAS, genome-wide association study; LB, leaf blotching; LD, linkage disequilibrium; LF, leaf firing; LRR, leucine-rich repeat; PCA, principal component analysis; QTL, quantitative trait loci; SAP, sorghum association panel; SNP, single-nucleotide polymorphism; TPR, tetratricopeptide repeat.
increases on the world market (www.fao.org/3/a-i4332e). As global temperatures increase as predicted by various climate change models (Tebaldi et al., 2006; Solomon et al., 2010), heat waves represent a major threat to world crop production (Teixeira et al., 2013; Rosenzweig et al., 2014). Therefore, incorporation of meaningful heat tolerance traits in breeding for environmentally resilient varieties is essential to sustain future agricultural productions under less favorable environments (Bita and Gerats, 2013).

In recent years, GWAS have been widely used for genetic characterization of large numbers of natural diversity panels of plant species for various traits (Morriss et al., 2013; Visioni et al., 2013; Rhodes et al., 2014; Adeyanju et al., 2015; Hayes et al., 2015; Pace et al., 2015). Many of the studies focus on simple agronomic traits that can be easily measured under field or controlled laboratory conditions (Higgins et al., 2014; Rhodes et al., 2014; Matsuda et al., 2015; Pace et al., 2015) and the biotic resistant traits that can be accurately scored (Wang et al., 2012, 2014; Berger et al., 2013; Zila et al., 2014; Gao et al., 2016). For other complex traits, such as abiotic stress tolerance or susceptibility, use of GWAS in the identification of marker–trait associations is still limited because of the difficulties posed by large-scale phenotyping of diverse populations under uniform environmental conditions.

Appropriate phenotyping for major heat tolerance traits in crop species are critical to identify genetic sources in breeding for heat-tolerant varieties as well as in the study of underlying genetic and molecular mechanisms of heat tolerance in crops (Powell et al., 2012). However, it is impractical to evaluate a large number of accessions of a given crop species effectively for heat tolerance traits under space-constricted growth chamber and greenhouse conditions, especially as crop plants are most sensitive to heat stress during rapid vegetative growth and reproductive stages and the majority of natural heat stress events occur at those stages.

Sorghum, the world’s fifth most important cereal crop in terms of production, is considered as a resilient crop because of its high tolerance to drought and high temperatures compared with other cereal crops. Nevertheless, substantial genetic variation exists among sorghum accessions for their abiotic tolerance levels (Kapanigowda et al., 2013; Abraha et al., 2015). Heat stress episodes occurring at critical reproductive stages often result in significant decreases in seed set of grain sorghum (Prasad et al., 2015). Our field studies have observed heat-induced reductions in plant height and photosynthetic area in some sorghum accessions when heat stress episodes occur during rapid vegetative growth stage, causing decrease in total biomass production of both grain and bioenergy sorghum. An understanding of the underlying genetic variation in the diverse responses of sorghum accessions to heat stress under field conditions not only can enhance our understanding of adaptation mechanisms in sorghum but may also help uncover the genetic basis of heat tolerance in other cereal crops through comparative genomic approaches.

In the current study, we performed a multiyear, field-based evaluation of the heat stress responses of sorghum leaves using 374 accessions of the US sorghum association panel during vegetative growth stage in three locations where heat wave events occur frequently throughout the sorghum growing season. Here, we report our findings on (i) the genetic variation for two major heat-responsive traits of sorghum leaf tissue in the sorghum association panel (SAP), LF, and LB, and ii) SNP markers significantly associated with LF and LB traits identified through the genome-wide association analysis.

**MATERIALS AND METHODS**

**Plant Materials and Environment Conditions**

The US SAP consisting of 374 accessions described in Casa et al. (2008) was used in this study. The SAP was planted in five environments using a completely randomized experimental design with or without replication. In 2013, the sorghum accessions were planted 10 d apart at two locations: Texas A&M AgriLife New Deal Research Station, New Deal, TX, (13TAEN) and the USDA–ARS Cropping Systems Research Laboratory (CSRL) in Lubbock, TX, (13CSRL) on 24 May and 3 June, respectively. In 2014, two replicates were planted on 30 May at the CSRL location (14CSRL). In 2015, two locations of a single replicate were planted: one was on 26 May at the CSRL location (15CSRL) and the other one was on 9 June at the Texas A&M AgriLife Chillicothe Research Station, Chillicothe, TX (15TAEC). The soil type at all locations is classified as Amarillo sandy loam (fine-loamy, mixed, superactive, thermic Aridic Paleustalfs). All experiment fields were furrow irrigated 1 wk prior to planting. For the CSRL location, the experimental fields were fully irrigated and fertilized via a subsurface drip system and kept under well-watered condition throughout the growing seasons by a daily scheduled irrigation of 5 mm of irrigation per day. The experimental fields at the 13TAEN and 15TAEC locations were furrow irrigated at both vegetative and early reproductive stages to provide ample soil moisture in addition to precipitation. No evidence of moisture stress was observed. Therefore, the phenotypic variations observed after a heat stress event on sorghum leaves of the association panels were considered as heat-responsive phenotypes associated with high-temperature stress during vegetative growth stages.

The meteorological data at the CSRL experiment location were recorded using an automated weather station (http://www.lbk.ars.usda.gov/WEWC/weather-pswc-data.aspx). The climatic environment at the 13TAEN location was similar to those of 13CSRL. Therefore, the 2013 weather data collected at CSRL were used for both 13CSRL and 13TAEN locations. The daily high-temperature data at the 15TAEC were the actual temperatures recorded by the local weather stations (http://www.accuweather.com/en/us/chillicothe-tx/79225/month/336018?monyr=6/01/2015).
Phenotyping for Heat Responsive Traits of Sorghum Developing Leaves

Natural heat stress events occur sporadically among years and locations and vary in intensity and duration. Here, the daily maximum air temperatures at the experimental field were used to define the heat stress levels. A major heat stress event was defined as a daily high air temperature reaching to 37°C or above in two or more consecutive days, whereas a moderate heat stress event was defined as a daily high air temperature reaching to 35°C or above in two or more consecutive days. In this field-based study, we evaluated heat responsive phenotypes of leaf tissues of sorghum association panels in accordance to the naturally occurring heat stress events during sorghum vegetative growth and flowering stages of the majority (~90%) of accessions in the association panel (Fig. 1). In general, the heat-induced tissue injuries on developing leaves of sorghum plants become distinctively visible a few days after the stress events have occurred and stay visible throughout the life time of those particular leaves being affected. Therefore, phenotyping ratings were performed 5 to 10 d after a major or moderate heat stress event. Specifically, phenotyping of sorghum plants were rated three times for the 13TAEN environment, twice for the 13CSRL environment, once for 14CSRL environment, and twice for both 15TAEC and 15CSRL environments.

We evaluated two major heat responsive tissue injury phenotypes of sorghum developing leaves in this study, LF, and LB. The LF phenotype is a type of heat-responsive phenotype resulting from heat-induced massive tissue injuries to developing leaves (Chen et al., 2010). The injured tissues die and desiccate quickly (Fig. 2A). The LB phenotype displays patches of discernible zebra-like discoloration zones on the leaf blade caused by heat-induced tissue injuries between veins on leaf tissues (Fig. 2B). The affected tissues are light green in color surrounded by green healthy tissues. The LF trait was scored as the number of leaves showing LF per plant. The LB trait was scored as the severity of the symptom showed on leaf blade of the most sensitive leaf of a given plant and rated on a 0-to-5 scale where 0 = no LB observed and 1, 2, 3, 4, and 5 represent less than 20, 40, 60, 80, and 80 to 100% leaf areas show LB phenotype, respectively.

Genotype Data

The genotype data of 310 accessions (SAP310) were obtained from the community resource of genotype-by-sequencing (GBS) dataset previously described (Morris et al., 2013) and the genotype data of the other 33 accessions (ADD33) were obtained from an in-house GBS data set for a group of sorghum germplasm assembled for breeding abiotic stress tolerance. Briefly, SNPs of those SAP310 were generated from GBS using the restriction enzyme ApekI digested libraries of SAP entries (Morris et al., 2013), while those of ADD33 GBS data set were generated using the restriction enzyme NgoMIV digested libraries. The GBS data of SAP310 consisted of 265,487 SNPs compared with the BTx623 genome reference. The GBS data of ADD33 represented ~60,000 SNP variants based on their alignment to BTx623 reference (www.phytozome.net). The GBS data of the two data sets (SAP310 and ADD33) were merged based on their common SNP positions to obtain a single data set for GWAS analysis used in this study. Imputation was performed on the
merged datasets using Tassel’s (v 5.2.18) built-in tool LD KNNi imputation plugin with high LD sites of 100 and number of nearest neighbors of 20 (Bradbury et al., 2007). After merging, the 343 entries with genotype data available were included in the calculation of the kinship matrix. The combined genotype data used in association analysis is provided as Supplemental File S1 (http://www.csrl.ars.usda.gov/psgd/sorghum/SuppTable%201_SorghumGWAS_Heatstress.txt). Principal components were calculated using the combined genotype data and the first two principal components were used to generate two-dimensional plots. Average phenotype scores across years and locations were calculated for each trait and the genotypes with score $\geq 1$ were highlighted in the two-dimensional principal component analysis (PCA) plots.

**Statistical Analysis**

Statistical analyses of phenotype data were performed using JMP 10.0 (SAS Institute, 2012). Based on the numerical nature of the phenotype data and trends observed, raw phenotype scores were used in the analyses. An analysis of variance was performed to assess the significance of contributions to phenotypic variation from genotype, environment, and genotype $\times$ environment interaction. All sources of variation were considered as random effects. The best linear unbiased predictor value (BLUP) for each SAP genotype–trait combination was calculated as described previously (Piepho et al., 2006). To partition the contribution of genetic and environmental components, we applied a random effects model, where both genotype entries and environment were considered as random effects, using restricted maximum likelihood method implemented through JMP 10.0 software (SAS Institute, 2012). All other variances that did not partition to either genotype or environment were pooled as residuals. Correlation matrix was calculated in R for the traits measured in each location using cor (mydata, 2) command and ggplot was used to generate a heatmap for the correlation matrix.

Analysis of the repeatability of phenotype scores to heat stress across environments was estimated using the following formula:

$$\text{Repeatability} = \frac{\sigma^2_G}{\sigma^2_G + \sigma^2_E + \sigma^2_R}$$

where $\sigma^2_G$ is the variance from genotype, $\sigma^2_E$ is the variance from environment, and $\sigma^2_R$ is the residual variance.
Association Analysis

Analysis of trait–marker association was conducted based on raw phenotyping data using a compressed mixed-linear model in GAPIT with three principal component covariates and the default VanRaden kinship matrix (Lipka et al., 2012). The linkage disequilibrium (LD) range was set to 3 to 30 kb for the analysis. This LD range value was chosen based on previous estimates that LDs for each sorghum chromosome can vary from 3 to 30 kb (Hamblin et al., 2006; Bouchet et al., 2012; Upadhyaya et al., 2012; Morris et al., 2013). A significance threshold of 5% false discovery rate (FDR) was applied to declare significant trait to genotype associations. The percentage variation explained by each of the associations was reported based on default parameters of the analysis package.

Candidate Genes near Associated Loci and Their Coexpression Networks

Genes colocalizing with or adjacent to the associated SNPs were determined using the sorghum reference genome assembly v2 on Gramene Mart (http://ensembl.gramene.org/Sorghum_bicolor/Info/Index). For most of the significant SNPs identified in this study, a genome scan spanning a region of 20 kb both upstream and downstream of the SNP was used to identify candidate genes. Subsequent functional annotations of the candidate genes were determined using the version 2.0 annotation of sorghum genome on phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and Gramene (http://ensembl.gramene.org/Sorghum_bicolor/Info/Index) websites. Putative SNP effects on amino acid changes were obtained from Gramene mart variant database (http://ensembl.gramene.org/biomart/martview/13d556c5e65da1b1f3aeeac89bd05b3a5). The coexpression networks for each of the candidate genes were retrieved from the MOROKOSHI sorghum transcriptome database (Makita et al., 2015; http://sorghum.riken.jp/morokoshi/Home.html).

RESULTS

Heat Stress Episodes and Phenotyping of Leaf Responsive Traits to Heat Stress Episodes

Although the timing at which the phenotypes were evaluated varied among years because of the sporadic nature of heat wave events, we were able to evaluate LF and LB heat response phenotypes at least once when all or majority of sorghum accessions were at vegetative stage and once after most of the accessions entered reproductive stage for most year–location environments (Fig. 1, indicated by arrows). In 2013, sorghum plants experienced multiple heat stresses where the daily air maximum temperatures reached 37°C or above at both locations. At the 13TAEN location, where sorghum plants of all accessions had entered rapid vegetative growth stages in late June, a total of 32 and 39 sorghum accessions showed different levels of LF and LB phenotypes, respectively, after a 4-d heat stress event. The number of accessions that showed LF increased to 39 after a brief heat stress event in mid-July when the majority of accessions were at late vegetative stages and some were about to enter flowering stage. The same stress event caused 42 sorghum accessions to show a LF phenotype and 29 to show a LB phenotypes at the 13CSRL location where all sorghum plants were at rapid vegetative growth stages (Fig. 1). The total number of sorghum accessions exhibiting LF and LB phenotypes in 13CSRL reached 45 and 38, respectively, after a heat stress event occurred in early August when the majority of sorghum accessions were either at late vegetative or flowering stages. In 2014 and 2015, sorghum plants grown at the CSRL location experienced a total 7 and 4 d of heat stress with the daily air maximum temperatures reached to 37°C or above compared with 12 d in 2013. Accordingly, the LF and LB ratings were either recorded once later in the season after a brief heat stress event (14CSRL) or combined as one rating as a result of lack of heat stress event for the rating recorded at vegetative stage (15CSRL). Sorghum accessions grown in Chillicothe experienced prolonged heat stress events during mid-to-late vegetative growth stages as well as at flowering stage. For the 15TAEC location, the LF and LB phenotypes were rated once in late July, when almost all sorghum accessions were still at vegetative growth stages, and another time late in August after the majority of accessions entered the reproductive stage (Fig. 1; Yr2015C). During the entire sorghum growing season, there were a total of 40 d where the daily air high temperature exceeded 37°C. The total numbers of sorghum accessions showing LF and LB phenotypes were 36 and 46, respectively.

We observed that both LF and LB phenotypes were the results of leaf tissue injuries induced by high temperatures. Leaf firing occurred most commonly on young developing leaves at the top of heat-sensitive plants, whereas LB often occurred on young as well as on rapidly expanding leaves. We observed that a few hours of high temperatures between 37 to 40°C did not cause visual tissue injury symptoms on mature leaves of sorghum plants but resulted in leaf rolling in a number of accessions. Therefore, the LF and LB phenotypes recorded in this study represented genetic variation in heat stress response for developing sorghum leaves.

Analysis of Phenotypic Variation

Notwithstanding the environmental variations, phenotype ratings for sorghum LF and LB traits were relatively consistent among years and environments. Figure 2A and 2B showed the distribution of sorghum association panel for LF and LB traits under the five environments, which were strongly skewed toward tolerance. Although developing leaves from the majority of sorghum accessions within this association panel exhibited high levels of tolerance to high temperatures under field conditions and showed little or no visible LF and LB, we identified a group of sorghum accessions that exhibited consistent sensitivity to heat stress over the period of the study. On average, the LF and LB phenotypes were observed in
~11% of sorghum accessions in four of the five environments (10–12% for LF and 10–13% for LB). Phenotype data from 15CSRL location were excluded in the calculation of average ratings because of lack of heat stress event during sorghum vegetative growth stages for most of the accessions (Fig. 1). The highly heat-sensitive accessions showed similar leaf tissue injury phenotypes under all environments analyzed. There were also some accessions that showed low level of tissue injuries under severe heat stress events but no LF- or LB-sensitive phenotypes under mild heat stress conditions. For LF trait, the LF ratings averaged 0.13, 0.18, and 0.23 per accession for three ratings performed at 13TAEN location and 0.17 and 0.24 for the two ratings performed at 13CSRL location. The LF ratings at 14CSRL averaged 0.24 and 0.20 for rep1 and rep2, respectively. The LF and LB ratings for 15TAEC location were 0.18 and 0.27, respectively.

Among heat-sensitive sorghum accessions, the average number of leaves displaying LF ranged from 1.5 to 2.3 and the average LB ratings ranged from 1.56 to 2.37. Analysis showed that genotypic differences in both the LF and LB traits were statistically significant with a relative high repeatability of 0.69 and 0.70, respectively. In addition, correlation analysis of LB and LF variables revealed a moderate correlation ($r = 0.49$) between these two traits among the sensitive accessions. The heat map in Fig. 2 displays the correlation of LF and LB over the five environments (Fig. 2C). Statistical analysis of the 10 most sensitive accessions of LF trait across all environments estimated that the BLUPs ranged from 3.6 to 2.1 (Table 1). The BLUPs for LB traits of the 10 most sensitive sorghum accessions ranges from 3.9 to 1.9 (Table 1). Furthermore, genotype specificity in the response to heat stress was also observed among the heat sensitive sorghums. Three of 10 sensitive accessions showed only LF or LB phenotype (Table 1), while the remaining seven accessions showed both LF and LB phenotypes (Table 1). It appears that there is no clear commonality among these sensitive accessions based on their pedigree, countries of origin, or working groups (Table 1).

Based on analysis of variance for both LB and LF, significant contribution can be attributed to different genotype entries but not to environment or genotype $\times$ environment interactions in this study (Table 2). Furthermore, on partition of the genotype and environment contributions to variance observed in this study, it is clear that genotype is the major source of variation for each of the phenotype evaluated. For LF, genotype differences accounted for 65% of variance, while for LB, genotype contributed 59% of the variation observed. The repeatability (0.70 and 0.69 for LF and LB, respectively) further confirms that observations over the 3 yr were consistent. These statistics further support the amenability of the raw data for genome wide analysis (Table 2).

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**Table 1.** The best linear unbiased predictor (BLUP) for leaf firing (LF) and leaf blotching (LB) traits of the 10 most heat-sensitive sorghum accessions for LF and LB among the 343 members of sorghum association panel. Significant single-nucleotide polymorphisms (SNPs) in bold font represent nonsynonymous missense variant.

<table>
<thead>
<tr>
<th>Traits</th>
<th>Genotype</th>
<th>Accession No.</th>
<th>BLUP</th>
<th>Origin</th>
<th>Working group</th>
<th>Significant SNP</th>
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</thead>
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<td>LF</td>
<td>RCV</td>
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Table 2. Variance components using restricted maximum likelihood (REML) method for leaf firing and leaf blotching heat-tolerance traits in 343 accessions from the US Sorghum association panel (SAP) over four growing environments.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Variance components</th>
<th>Variance ratio</th>
<th>Variance estimate ($\sigma^2$)</th>
<th>Standard error</th>
<th>Percentage of total (contribution to variance)</th>
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<td>Leaf firing</td>
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<td>Environments</td>
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<td>0.000</td>
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</tbody>
</table>

Genotype Data Analysis

The genotype data of 343 sorghum accessions were merged based on their common SNP positions, resulting in a total of 13,987 SNPs. Genotype data from four sorghum accessions had >40% missing data after merging and imputation and were excluded from the association analysis. Therefore, the association analysis was performed on a total of 339 accessions using 13,987 SNPs. The SNPs were distributed across all 10 chromosomes (Supplemental Fig. S1A). We estimated the relative kinship and found 66.5% of the kinship coefficients ranged from 0 to 0.05, indicating that most accessions have no or only weak genetic relationship with the other genotypes. Heat map derived from kinship relationship is provided in the supporting files (Supplemental Fig. S1B). Principal component analysis demonstrated that the top two components clearly separated these accessions into four subgroups, which is consistent with the previous reports for this association panel (Casa et al., 2008; Zhao et al., 2016). The majority of sorghum accessions fall into four races: Guinea, Durra, Kafir, and Caudatum, respectively (Fig. 3). Although the first two components of PCA are able to explain >25% of the total genetic variance, ambiguous clustering patterns are still occasionally observed and are classified as mixed group (Fig. 3). Linkage disequilibrium analysis across the genome and individual chromosomes indicated that the rate of LD decay was similar across chromosomes. The distance of reaching the LD threshold of $r^2$ value of 0.2 ranged from 1 to 10 kb on different chromosomes. Across the whole genome, the average distance of reaching LD $r^2 = 0.2$ was ~7 kb (Supplemental Fig. S2). At 10 kb, the $r^2$ value averaged 0.15 for all 10 chromosomes and ranged <0.1 to 0.2 across different chromosomes (Supplemental Fig. S2). The $r^2$ values dropped to 0.10 after 30 kb for most of the chromosomes. These results are comparable with those previously reported (Bouchet et al., 2012; Morris et al., 2013; Upadhyaya et al., 2012) and indicated that markers in 10 to 30 kb range are weakly linked.

Association Analysis

Genome-wide association of genetic variation with LF and LB ratings for heat tolerance in sorghum association panel was analyzed using the compressed mixed-linear model in GAPIT. Using a FDR significant threshold of $\leq 0.05$ for trait-genotype association, we identified 14 SNPs significantly associated with at least one trait under two or more environments (Fig. 3; Table 3). Among the SNPs identified, nine loci were significantly associated with LF trait, four loci were significantly associated with LB trait, and one locus was significantly associated with both LF and LB traits (Table 3). The Manhattan plots displayed in Fig. 4 represented the genome-wide distribution for the association of the 13CSRL LF and 15TAEC LB traits on 10 sorghum chromosomes. Two SNPs, one on chromosome 2 (S2_76939617; $P = 1.15 \times 10^{-7}$) and another on chromosome 4 (S4_34147673; $P = 3.13 \times 10^{-7}$) showed significant association with LF trait in 13CSRL location (Fig. 4A). For LB trait, two significant SNPs, one on chromosome 6 (S6_42792390; $P = 4.28 \times 10^{-8}$) and one on chromosome 9 (S9_49702613; $P = 7.28 \times 10^{-8}$), were identified in 15TAEC environment (Fig. 4B). The genome-wide distributions for the association analysis of LF and LB traits on the 10 sorghum chromosomes under all other environments can be found in Supplemental Fig. S3. Quantile–quantile plots for LF and LB traits analyzed over all year and location time points are also provided as Supplemental Fig. S3.

The identification of the 14 significant trait-associated SNPs in at least two or more environments in this study indicates high repeatability and quality of the phenotyping data. To examine the repeatability of the specific marker–trait association, we overlaid Manhattan association plots across years and locations for three quantitative trait loci (QTL) that showed significant association with LF, LB, or both LF and LB traits (Fig. 5). Here, in addition to QTL identified with the threshold of $p$-value $\leq 0.05$ reported in Table 3, we also included some year-locations that show similar associated peaks with FDR values $\geq 0.05$. At this threshold, significant associations of SNP S2_76939617 were identified for the LF trait measured at five different environments, years, and location time points (Fig. 5A). Likewise, significant associations of a SNP on chromosome 9 (S9_49702613) with LB traits were identified for the four different year and location time points (Fig. 5B). Significant associations of two SNPs on chromosome 4 (S04_34147673 and S04_59762508) with both LF and LB traits were identified repeatedly across locations and years (Fig. 5C).

Candidate Genes Colocalized with Associated Single-Nucleotide Polymorphisms

The majority of the significantly associated SNPs identified in this study were found either within an annotated gene or within a few hundred nucleotides upstream or downstream of an annotated gene (Table 3). The only exceptions were the two significant SNPs identified on chromosome 7 (S7_16734813 and S7_16734910), which
were found to be intergenic variants between the two closest genes, Sb07g009510 and Sb07g009520, which are ~47.5 kb upstream and 33.3 kb downstream of these SNP markers, respectively.

Candidate genes associated with SNPs were examined for their annotated functions. We found that the candidate genes encode several classes of proteins with known or predicted functions in biotic, abiotic, and wounding response pathways (Table 3), including F-box domain and leucine-rich repeat (LRR)-containing protein, zinc finger (CCCH-type) family and RNA recognition motif-containing protein, receptor-like protein, NAC domain containing protein, helix-loop-helix DNA-binding domain containing proteins, glutathione S-transferase TAU, Rad21/Rec8-like protein, Sec1 family transport protein, pentatricopeptide, eukaryotic aspartyl protease family protein, and auxin-responsive protein (Table 3). The predicted gene functions for candidate genes associated with significant SNPs were listed in Table 3 along with putative allele effects of SNPs on amino acid coding sequencing changes.

In addition, we examined the coexpression networks for each of the associated candidate gene identified in this study. The top 20 genes identified in the coexpression networks were listed in Supplemental Table S1. We found that several coexpression networks of candidate genes have been reported to be involved, foremost is the regulation of heat shock response, plant–pathogen interactions, and hormone responses among networks identified (Supplemental Table S1). Notably, the coexpression network for heat shock response provides additional evidence that the candidate genes may be involved in heat stress response. Additionally, the identification of coexpression network for plant pathogen interactions could be related to the similarity of the process of LF or LB with hypersensitive response during pathogen attack, which likely involved related network of genes.

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**Fig. 3.** Principal component analysis of sorghum association panel used based on the 13,987 common single-nucleotide polymorphisms identified from genotype data of two public sources used in this study. Each entry is represented by circle and grouping in accordance with membership to previously reported major races of sorghum were indicated by shaded oblong shape. The five major races of sorghum were resolved in this study as shown. The heat sensitive lines identified in Table 1 and other heat sensitive lines with a BLUP value >1 are indicated as closed circles or triangles and by different colors.
we also observed genetic specificity of heat-sensitive sorghum accessions in their responses to heat stress. Among the 10 most sensitive accessions for either LF or LB traits, seven overlapped and showed both LB and LF phenotypes, while three consistently showed either a LB or a LF phenotype. Such genotypic variation in response to a heat stress may indicate underlying similarities and differences in the regulation of heat stress responses of sorghum developing leaves among these accessions.

Given the variable nature of the heat stress events that occurred among seasons and locations (Fig. 1) as well as differences in planting dates and in relative humidity of the environments, the high repeatability for both LF (0.70) and LB (0.69) traits obtained in this study indicates that phenotypic variations observed among panel members are contributed largely by their genetic differences. The results indicate that the phenotypic data gathered are generally of high quality and suitable for subsequent genome-wide analysis. Analyses of variance for both LF and LB also indicate significant contribution of genotype variation to the observed difference in both LF and LB heat stress tolerance traits, whereas environment variation and genotype × environment interaction contribute

**DISCUSSION**

**Phenotypic Variation and Correlations**

Sorghum is considered a heat-tolerant species among cereal crops. Nevertheless, studies have shown genetic variations for heat tolerance of both vegetative and reproductive tissues exist among sorghum accessions. The present study uses a GWAS approach to identify genes or gene variants associated with tolerance or susceptibility of sorghum leaf tissues to heat stress under field conditions. Within the panel used in this study, ~90% of the accessions flower 50 to 75 d after planting (Casa et al., 2008). Although both pre- and postanthesis heat sensitivity of photosynthetic tissues are important factors contributing to grain yield losses, in this study we evaluated two heat-responsive traits of sorghum developing leaves associated with preanthesis heat stress. Although there were differences in the timing and extent of heat stress events among years and locations (Fig. 1), the developing leaves of ~10% of sorghum accession showed consistent sensitivity to heat stress (either LF or LB or both), while the remaining 85% accessions showed consistent tolerance (no LF and LB) (Fig. 2).

<table>
<thead>
<tr>
<th>SNP physical position</th>
<th>Gene containing or adjacent to SNP</th>
<th>Distance to SNP†</th>
<th>FDR values‡</th>
<th>Trait (environment)$§$</th>
<th>Allele</th>
<th>Allele effect¶</th>
<th>$R^2$#</th>
<th>Annotated gene and function</th>
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<td>G/C</td>
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<td>0.46</td>
<td>0.41</td>
</tr>
</tbody>
</table>

† Physical distance (bp) from the annotated gene to the significant SNP. Plus sign (+) indicates upstream of the gene from the annotated starting basepair; minus sign (−) indicate downstream of the gene from the last basepair of the annotated gene.

‡ Lowest p-value or false discovery rate (FDR) value.

§ Numbers in parenthesis indicate the numbers of environments under which the marker–trait association were significant.

¶ Original rating score for the corresponding trait indicated in the table.

# Proportion of total line mean variance explained by SNP as computed by GAPIT.
little to the variation observed (Table 2). These results suggest that the genetic architectures underlying the heat stress responses of sorghum developing leaves observed in this study maybe relatively controlled by few genes.

**Genotype Data**

The availability of genotype data sets (resulting from genotype × sequencing analysis) necessitates the need to merge GBS data sets for use in association analysis to find the linkages between the markers and the phenotype. The use of merged data for GWAS has been also successfully applied in a number of reports on association analysis (Peiffer et al., 2014; Boyles et al., 2016; Zhao et al., 2016). In this report, the merged SNP data and SNP density was reduced compared with the two original data sets because of the exclusion of the rare variants from each of the GBS datasets. Thus, we have evaluated possible effect of SNP density reduction on our association analyses. Principal component analysis of population structure of the 339 the genotypes have shown race and working group clusters (Supplemental Fig. S1A; Fig. 3), similar to those of previous reports (Casa et al., 2008; Morris et al., 2013). We also compared marker–trait associations between a subgroup of 310 accessions with 265K SNPs and the 339 panel with 14K SNPs evaluated in this study and found that the reduced number of SNPs did not affect the outcomes of the association analysis.

![Fig. 4. Manhattan plots show association result for leaf firing (LF) and leaf blotching (LB) traits in a panel of 339 accessions based on 13,987 common single-nucleotide polymorphisms (SNPs). The x-axis represents the physical map locations of the SNPs and the y-axis represents the −log base 10 P-values.](image-url)
Association Analysis

Association analysis on LF and LB traits shows significant marker–trait association for each of the locations. Many of the significant SNPs identified for LF and LB traits are either colocalized with an annotated gene or located within a few hundred basepairs upstream or downstream of an annotated gene (Table 3). These findings are comparable to previous reports in GWAS focusing on abiotic stress (Visioni et al., 2013; Kumar et al., 2015; Matsuda et al., 2015; Pace et al., 2015; Bac-Molenaar et al., 2016).

The moderate correlation between LF and LB traits ($R^2 = 0.488$; Fig. 2C) obtained is consistent with our field observation as well as with association analyses reflected by the two significant SNP markers associated with both LF and LB traits (Table 3). We observed that some heat-sensitive lines showed both LF and LB phenotypes after a heat stress event. Leaves with severe LB on these plants could also develop into LF under severe heat stress conditions (high intensity, long duration, or both). However, the observation that some sensitive genotypes consistently show only LF on young developing leaves or LB on rapid expending leaves, while a few others show both LF and LB phenotypes suggest genotype specificity in their response to heat stress among sensitive accessions within the selected sorghum panel.

The overlaied Manhattan association plots for three QTL chromosome regions demonstrate the reproducibility of the specific marker–trait association (Fig. 5) across years and locations. The associated variant SNPs with LF and LB identified in this study tagged susceptible reaction; conversely, it is inferred that SNP genotype found in tolerant accessions are candidate markers for heat tolerance. We compared the significant SNPs and genomic coordinates identified in this study with those of previously mapped in sorghum for agronomic traits or stress related traits (Morris et al., 2013; Mantilla Perez et al., 2014; Zhang et al., 2015; Boyles et al., 2016) and results revealed little overlaps over the genome regions. This suggests that most of the variation associated with LF and LB heat responsive traits revealed in this study may be novel and is influenced through rare alleles present in the panel. Further investigation on the genetic network regulating these traits and their functional relationship could be useful in molecular breeding for enhanced heat tolerance in sorghum and possibly in other cereal crops as well such as corn and wheat.

Candidate Genes

In the present study, candidate genes for 13 unique SNPs significantly associated with LF and LB heat-tolerant traits have been identified. The associated regions contain candidate genes that belong to several categories based on biological function such as transcription factors, kinases, signal transduction, disease resistance, transporters, and antioxidants (Table 3). Among the genes identified for SNP markers significantly associated with LF traits, two encode proteins with receptor-like functions. The Sb09g026470 (Sb09g026470_S9_55739930 SNP, missense variants) encodes a putative membrane-localized receptor-like protein containing...
toll-like and LRR domains. The \(S_b06g015470\) gene (SNP \(S_6\_{42792390}\)) encodes a purple acid phosphatase 22. Homologs of these sorghum genes have either been shown or proposed to be involved in the receptor-signaling pathway of heat stress (Kaida et al., 2010; Antonyuk et al., 2014) and in the sensing of various environmental stimuli and in the regulation of signal networks in response to biotic and abiotic stresses (Osakabe et al., 2013; Park et al., 2014). The association of sorghum receptor genes with LF traits uncovered in this study suggests a role of these proteins as signaling receptor, sensing the environmental temperature changes, and regulating the downstream responses to the detected heat stress. Further studies will be performed to investigate the function of this class of proteins in temperature sensing in plant systems.

We have identified three LF trait associated genes that their homologs are known in the regulation of abiotic stress responses including heat stress response. The \(S_2\_{76939617}\) SNP identified on chromosome 2 (Table 3; Fig. 5A) carries a nonsynonymous missense variant within the \(Sb02g043180\), a gene encoding a putative Cysteine3Histidine (CCCH)-type zinc finger family protein/RNA recognition motif-containing protein. The other two SNPs, \(S_5\_{5778642}\), and \(S10\_{4009880}\), are located 1857 and 938 bp downstream of \(Sb05g024540\) and \(Sb10g004500\) genes. The \(Sb05g024540\) encodes a putative NAC protein and the \(Sb10g004500\) encodes a putative transcription factor of the bHLH family protein. Studies have shown that CCCH-type zinc finger and NAC proteins are regulators of plant responses to abiotic stresses and CCCH-type zinc finger proteins are involved in messenger RNA metabolism of abiotic stress responsive genes (Peng et al., 2012; Jan et al., 2013; Nuruzzaman et al., 2013; Castilhos et al., 2014; Golldack et al., 2014; Bogamuwa and Jang, 2014, 2016; Liu et al., 2015). The three genes also have a role in regulating heat stress responses in plants. It has been reported that the NAC transcription factors positively regulate the basal and acquired thermotolerance in plants and plays a key role in the survival of the plant under heat stress conditions (Shahnejat-Bushehri et al., 2012; Nakashima et al., 2014). \(Arabidopsis\) homologs of \(Sb05g024540\) and \(Sb10g004500\) genes are both downregulated by heat stress (Penfield et al., 2005). Furthermore, gene coexpression analysis reveals genes of common functions within their gene networks, specifically genes known to be involved in heat stress responses such as heat-shock proteins, kinases, and phospholipase (Supplemental Table S1). The significant association of LF trait with these gene variants in sorghum suggests similar roles of these transcription factors in the heat stress responses in sorghum leaves.

Another two SNP markers significantly associated with LF trait identified on chromosome 9 (\(S9\_{49644908}\) and \(S9\_{55739930}\)) are both missense variants (Table 3) of \(Sb09g020350\) gene (\(S9\_{49644908}\)) encoding a putative tau class glutathione S-transferase (GSTU) TAU 8. This class of glutathione S-transferase is plant specific, and involved in detoxification of herbicides in crops and weeds (Benekos et al., 2010; Wang and Yang, 2011), and plays an important role in protecting plants against various types of abiotic stresses (Jha et al., 2011; Rezaei et al., 2013; Sharma et al., 2014; Xu et al., 2015). Another SNP identified on chromosome 9 (\(S9\_{6943724}\)) for LF trait is located 765 bp upstream of \(Sb09g005430\) gene, which codes an auxin-responsive AUX/IAA15 protein involved in regulation of transcription in auxin-activated signaling pathways. In addition, there are two heat shock protein genes (\(Sb09g005570\) and \(Sb09g005580\)) located downstream of \(S9\_{6943724}\).

For LB trait, the present study has identified three significant SNPs that are solely associated with this phenotype in the sorghum association panel. The SNP identified on chromosome 2 (\(S2\_{6474843}\)) is a missense polymorphism in \(Sb02g005460\), encoding a putative peptidase S8/S53 and is highly homologous with \(Arabidopsis\) subtilase family proteins. The nearby \(Sb02g005450\) gene is located 1284 bp downstream of the SNP marker and encodes a putative tetratricopeptide repeat (TPR)-like superfamily protein pentatricopeptide. The TPR protein is reported to regulate gene expression at the RNA level and in stress responses and hormone signaling ( Sharma and Pandey, 2015). In \(Arabidopsis\), a TPR family protein containing E3 ligase can remove damaged proteins under extreme temperature conditions, which apparently result in increased electrolyte leakage from leaf cells (Yan et al., 2003). The \(S9\_{49702613}\) SNP associated \(Sb09g020390\) gene encodes a putative member of F-box/RNI and LRR domain containing protein families. Genes in these families are involved in responses to various types of abiotic stresses such as salt, drought, desiccation, and cold stresses in plants (Jain et al., 2007; Song et al., 2015). F-box/LRR gene is also reported in the QTL region of spikelet fertility under heat stress at flowering in rice (\(Oryza sativa\) L.) (Ye et al., 2015). The SNP marker \(S7\_{16734910}\) is an intron variant of \(Sb07g009510\) gene encoding a putative transducin/WD40 repeat domain-containing protein on chromosome 7. A large number of WD40-repeat proteins are involved in a variety of critical functions in plant growth and development processes as well as in abiotic stress responses of plants (Guerriero et al., 2015; Kong et al., 2015; Sharma and Pandey, 2015; Liu et al., 2016).

Notably, the two SNPs significantly associated with LB and LF traits are both located on chromosome 4. \(S4\_{34147673}\) is an intron variant of \(Sb04g015010\) gene that encodes a putative Sec1/Munc18 family transport protein. \(S4\_{59762508}\) is a missense variant of \(Sb04g029680\) gene that encodes a putative eukaryotic aspartyl protease family protein. The Sec1 family transport proteins are known to be involved in a variety of vesicle transport processes and performing essential functions in SNARE-mediated membrane fusion in plants, animals, and microbes (Bretscher and Clotworthy, 2007; Weber-Boytov et al., 2011, 2016; Karnik et al., 2013). A mutation of the \(sec1\) gene in yeast and \(Dictyostelium discoideum\) renders cell sensitive to high temperature for growth and defective in vesicle secretions (Tomeo et al., 1997; Bretscher and Clotworthy, 2007; Weber-Boytov et al., 2011, 2016; Karnik et al., 2013).
2007; Weber-Boyvat et al., 2016). In the present study, we observe a significant association of a Sec1 variant with LF and LB leaf tissue injury traits in sorghum. Our result suggest that maintaining essential function of Sec1 is critical for the thermotolerance of cell membrane under high-temperature conditions. For aspartyl protease, although it has an essential role in plant resistance to fungal pathogens and plant defense systems (Li et al., 2016), information about its role in abiotic stress in plants, specifically in heat stress response, is scarce and further studies are needed. Overall, with the exception of the unknown function gene Sh09g0050000, all other candidate genes are grouped in gene families that have been reported for their involvement in some aspects of abiotic stress tolerance in plants. Further investigations on the functions of these candidate genes in heat stress response and temperature sensing in sorghum are essential for validating the usefulness of these markers in marker-assisted selection for enhancing heat tolerance of photosynthetic leaf tissues in plant systems.

CONCLUSIONS

The current study is the first effort to apply GWAS to identify loci underlying heat tolerance or sensitivity in sorghum leaf tissues during vegetative growth stage. While sorghum is considered a heat tolerant cereal species, susceptibility to heat stress based on leaf responsive traits such as LF and LB phenotypes occur in the species. Results showed consistent ratings for both LF and LB traits despite variation in heat stress events among locations and years. In total, we have identified 14 SNP markers significantly associated with LF or LB or both LF and LB traits in sorghum. These associated variants with LF and LB or both LF and LB were linked to the susceptible reactions of sorghum developing leaves and could serve as candidate DNA markers for selecting heat tolerance. Candidate genes were found to be either directly or linked to a biological pathway involved in plant stress responses, some of which have been implicated to have direct roles to a biological pathway involved in plant stress responses, and therefore involved in abiotic stress-based heat tolerance. The findings of this study provide new knowledge on the genetic control of heat stress response and tolerance in sorghum leaves. The results of the current study also provide foundational information in the identification of molecular markers for molecular breeding of enhanced heat tolerance of photosynthetic tissues in breeding program.

Supplemental Information Available

Supplemental File 1: Genotype data of the 339 sorghum accessions used in the association analysis can be accessed at http://www.csrl.ars.usda.gov/psgd/sorghum/SuppTable%201_SorghumGWAS_Heatstress.txt

Supplemental Table 1: List of the top 20 genes in the co-expression networks of the genes associated with SNPs peaks identified for both leaf firing and blotching in this study. The co-expression networks were obtained from Morokoshi, Sorghum transcriptome database (sorghum.riken.jp) as described in Makita et al., 2015.

Supplemental Fig. 1A, B: Distribution of SNPs (A) across chromosomes and (B) heat map of the kinship matrix estimated from the sorghum genotype data used in this study.

Supplemental Fig. 2. Linkage disequilibrium decay across all 10 chromosomes within the 339 entries in sorghum association panel used in this study, where y-axis is the average r2 value and x-axis is the physical distance between the markers.

Supplemental Fig. 3A–R: Manhattan plots for sorghum developing leaves’ responsive traits to heat stress, leaf firing and leaf blotching across years and locations.

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References


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