ABSTRACT

Early season cold tolerance during stand establishment at cool soil temperature of 54°F to 59°F (12 ºC to 15ºC) is a key agronomic trait for warm season cereal crops such as sorghum. Sorghum (Sorghum bicolor L. [Moench]), lacks cold tolerance and is adversely affected by cool temperatures during germination, emergence/early seedling growth and at reproductive stages. To better understand the genetics of cold tolerance in sorghum, discovery of germplasm sources, hybridization and mapping of quantitative trait loci (QTL) with focus on early season cold tolerance traits were achieved. Five Chinese Kaoliang germplasm, three novel chemical mutants and two recombinant inbred populations (RIL at F7 generation) were developed. The parents, mutants and RIL populations were evaluated for cold germinability in controlled environment, field emergence, seedling vigor and biomass in multi locations during early season planting. Focusing on F7 RIL population of RTx403xPI567946, several QTL were localized in chromosomes 1, 4, 7 and 9 and identified to be associated with improved early field emergence and vigor. Particularly interesting are DNA markers for QTL localized in chromosome 9 that gave highest contribution to phenotypic variation in the population. Notably, this work demonstrated that the cold tolerance trait, specifically field emergence was not strictly associated with high tannin trait and non-tannin Kaoliang lines are valuable germplasm source of cold tolerance for sorghum.
INTRODUCTION

Germination and seedling establishment are adversely affected by cool soil and air temperatures in most crops, but the effect of the stress is more pronounced for warm season cereals such as sorghum. The inability to germinate prevents emergence resulting in poor stands, and growth of seedlings are hampered, delaying sowing, planting and crop growth. Low temperature induced inhibition of germination and emergence in the field is a common problem encountered in crops that originate from warm dry conditions which were bred to adapt to grow in temperate and cooler conditions specifically during early season sowing.

Sorghum is known for its drought tolerance and adaptation to high temperature. However, most varieties under cultivation are vulnerable to cooler conditions during stand establishment at early season planting from April to May in majority of the US sorghum belt. Stand establishment and early season vigor of sorghum is adversely affected by air and soil temperatures below 60ºF (15°C) during germination, emergence and early seedling growth (Yu and Tuinstra, 2000). Robust cold tolerance is highly advantageous in sorghum since it could facilitate 2 cropping cycles (an early-early plus regular season plantings). The trait could also be expected to stabilize and increase yield by establishing excellent crop stand and maintaining high plant density starting at the critical sowing period. Cold tolerance is also considered as a key trait needed for other types of sorghum; including biomass and forage types.

Sorghum is diverse and variation for cold tolerance have been reported and identified within its germplasm pool. Reports have indicated that possible sources of cold tolerance are landraces that have adapted and evolved in the temperate regions of China (Qingshan and Dahlberg, 2002). Previous reports have shown that Chinese landraces known as kaoliang exhibited higher seedling emergence and improved seedling vigor under cool conditions compared to select US hybrids and elite inbreds (Franks et al., 2006). However, most of these landraces also harbor poor or undesirable agronomic
traits. Thus it is deemed important to dissect the cold tolerance trait and devise an effective and efficient means to transmit the trait to elite parental lines of sorghum.

Previously, a Chinese kaoliang, Shanqui Red, has been reported as a parental source of early season cold tolerance trait (Knoll et al, 2008). To facilitate transfer of the early season cold tolerance, genetic mapping of genome regions associated with cold tolerance through identification of QTL was carried out (Knoll et al., 2008). Three markers associated with the QTL have been successfully used to verify introgression of cold tolerance in two populations using Shanqui Red as the common donor for cold tolerance (Knoll et al., 2008b). However, a drawback with the use of this population is that the parental source of cold tolerance (SQ red) harbors tannin and results indicate possible linkage between cold tolerance and the tannin gene(s).

As early season cold tolerance trait is a complex trait controlled by a number of genes, it is important to identify various sources of tolerance to effectively combine favorable alleles and avoid linkage drag. This approach could serve as most effective way to produce elite sorghum lines with stable and robust early season cold tolerance. Here we summarize the research conducted in our laboratories on genetic dissection of early season cold tolerance using two recombinant inbred populations, focusing on results from RTx430 x PI610727, the latter (PI610727) serving as a new source of early season cold tolerance.

MATERIALS AND METHODS

Germplasm and screening for early season cold tolerance

Five Kaoliang germplasm identified as new sources of early season cold tolerance are shown in Table 1. From the new sources, two advanced recombinant inbred population were established: RTx430 x PI610727- “Popn. 1” and BTx623 x PI 567946-“Popn. 2”.

In this report we focus on Popn. 1. The female parent, RTx430 is a widely adapted inbred and an important pollinator/restorer line of sorghum (Miller, 1984). PI610727 (also known as Gaigao liang), a land race selected from previous screening of a group of Chinese germplasm for early season
cold tolerance because it lacks pigmented testa and has no tannins (Franks et al, 2004). F₁ hybrids were developed by hand emasculation of RTx430 and subsequent pollination with PI610727. A confirmed F₁ plant was self pollinated and 200 seeds were advanced to the F₂ generation. Individual F₂ plants were selfed and advanced by single seed descent to the F₆. Seeds from each line were bulked after the F₆ generation. The mapping population consisting of 174 F₇ recombinant inbred lines had been deposited and will be made available for distribution through the USDA Agricultural Research Service Genetic Resource Information Network (GRIN).

Table 1. List of germplasm with stable early season cold tolerance.

<table>
<thead>
<tr>
<th>PI #</th>
<th>Chinese Name</th>
<th>Feature</th>
<th>Applications*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 610727</td>
<td>Gaigao liang</td>
<td>Excellent cold germinability, non-tannin</td>
<td>Parent for population, possible seed parent line (cms female)</td>
</tr>
<tr>
<td>PI 567946</td>
<td>Hongkezi</td>
<td>Excellent cold germinability &amp; vigor, non-tannin</td>
<td>Parent for population, possible seed parent line (cms female)</td>
</tr>
<tr>
<td>PI 563976</td>
<td>Dazhong jiao</td>
<td>Excellent cold germinability &amp; vigor, heterotic effect</td>
<td>Possible seed parent line (cms female)</td>
</tr>
<tr>
<td>NSL 51071</td>
<td>Kaoliang</td>
<td>Excellent cold germinability &amp; vigor, heterotic effect</td>
<td>Possible seed parent line (cms female)</td>
</tr>
<tr>
<td>PI 568016</td>
<td>Niuzheng zui</td>
<td>Excellent cold germinability &amp; vigor, heterotic effect</td>
<td>Possible seed parent line (cms female)</td>
</tr>
</tbody>
</table>

*cms- cytoplasmic male sterile

Parents and RI lines were screened for differences in cold and optimal temperature germinability using the procedures recommended by the Association of Official Seed Analyst (AOSA 1999). Each entry is represented by three replications and all experiments were repeated at least twice. Briefly, high quality seeds of each RI line harvested were used in the study. For cold and optimal germinability, 25 seeds were sown in polystyrene Petri dishes lined with filter paper moistened with sterile distilled water. Seeds were allowed to incubate/germinate at a constant 12°C(cold) for cold
germination and for optimal germination at 30°C for 8h alternate with 20°C for 16h, in the dark, in separate controlled temperature chambers. Germination was determined visually based on protrusion of radicle to approximately 1mm length. Final germination was counted at 4 and 7 days after sowing for optimal and cold temperatures, respectively.

To determine variation in field emergence, the RILs and parents were sown in 3 x 0.4 m plots at the USDA-ARS farm in Lubbock, TX (101° 90’ west longitude; 33° 59’ north latitude) and at Texas Agrilife farm at New Deal, TX (101° 82’ west longitude; 33° 69’ north latitude) in April, 2008. Multi locations trials for Popn 2 were conducted in 2010 and 2011 in Lubbock, Kansas, and Hereford, TX. Plots were uniformly irrigated using a drip system after sowing. Each RIL entry and parents were represented by three replications and experiments were laid in a randomized complete block design. Emergence was determined using counts of number of seedlings that have emerged at 30 days after sowing. Seedling vigor was scored based on previous rating scale by Maiti et al (1981); where 1-represents the most vigorous and robust seedlings and 5 – represents poor vigor, primarily based on size and physical appearance of the seedlings.

**DNA extraction, genotyping and linkage map construction**

Leaf samples from bulked plant samples (3 plants) of parents and RILs were harvested and used for genomic DNA extraction. DNA was extracted using a high throughput method previously described (Burow et al, 2009).

Genotyping was conducted through an analysis of simple sequence repeat markers or microsatellites by polymerase chain reaction. Microsatellite markers including LBK_SEAMs Xtp, Xcup, and Xgap series were used for the studies. Amplification of microsatellites was carried out by polymerase chain reaction as described in previous paper (Burow et al., 2009). Products were separated using an ABI 3130xl machine or by agarose gel electrophoresis (3.0% using SFR, Ameresco). For agarose gel separation fragment were visualized after ethidium bromide staining and gels were documented and archived using ChemImager. The genotype data for each marker was tested
by chi-square test to detect segregation distortion. Majority of the genotype data (99%) for this study were generated using ABI 3130xl.

The construction of linkage map was performed using software MAPMAKER/EXP v.3.0b (Lander et al., 1987). The distances between DNA markers in each linkage group were estimated using the Kosambi mapping function (Kosambi, 1944). A minimum LOD score of 3.0 and cM distance of 50 was applied in the construction of the linkage map. Linkage group were aligned to previously established maps by, Bhattaramakki et al., 2000; Menz et al., 2002 and Yu and Huang, (2006). The linkage groups were then numbered based on chromosome designation from Kim et al., (2005).

QTL Mapping

Statistical analysis from phenotypic evaluations was carried out using Excel and SAS. Germination data from the optimal and cool temperature analysis were transformed using the arcsine function to improve homogeneity of error variances.

Identification of QTL for six traits was performed using QTL Cartographer version 2.5 (Basten et al., 2003). Significant QTL were identified on the basis of composite interval mapping (CIM) module using the experiment wise threshold level determined by computing 1,000 permutations (Churchill and Doerge, 1994). QTL effects are expressed relative to the RTx430 allele. A positive additive effect indicates an increase in value for the trait if RTx430 allele was present for that QTL. A negative effect then indicates that the RTx430 allele would reduce the value for the trait. Graphic representation of linkage groups and QTL were obtained using Map Chart 2.1 (Voorips et al., 2004).
RESULTS

Screening for early season field emergence and vigor

The parents (RTx430 and P610727) used in the study differ on the basis of germinability at 30 and 12°C, field emergence and vigor (Table 2). In general, the parents exhibited intermediate to low seedling vigor. The information on soil and ambient air temperatures recorded daily during early season planting from April 1 to 30, 2008 and mean temperatures during the early season planting conducted in this study was cool for both soil and ambient air (14.9 and 17 °C).

The recombinant inbred progenies exhibited mean values for cold and optimal germinability of 73% and 88%, respectively (Table 2). The data between optimum and cold temperatures germinability; field emergence from two locations displayed significant positive correlations with each other. Lower positive correlations were found between cold /optimal germinability data and field emergence at both locations. Vigor was negatively correlated to germinability as expected due to the trend of the scoring system applied. However, vigor ratings between the two locations tested was positively correlated to each other, with r value of 0.45 (data not shown).

Table 2. Phenotypic analysis for early season cold tolerance of parents and progenies of Popn 1.

<table>
<thead>
<tr>
<th>Traits</th>
<th>Parents RTx430</th>
<th>PI610727</th>
<th>RIL population Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Germ @12°C</td>
<td>38.67</td>
<td>98.67</td>
<td>73.13</td>
<td>28% to 98%</td>
</tr>
<tr>
<td>%Germ@30°C</td>
<td>60.00</td>
<td>100.00</td>
<td>88.23</td>
<td>48% to 100%</td>
</tr>
<tr>
<td>%Emerge_CSRL</td>
<td>16.00</td>
<td>68.00</td>
<td>31.85</td>
<td>2% to 77%</td>
</tr>
<tr>
<td>%Emerge_NewDeal</td>
<td>9.33</td>
<td>68.67</td>
<td>34.15</td>
<td>4.47% to 79.33%</td>
</tr>
<tr>
<td>Vigor_CSRL</td>
<td>3.88</td>
<td>2.50</td>
<td>3.03</td>
<td>1.25 to 4.25</td>
</tr>
<tr>
<td>Vigor_NewDeal</td>
<td>4.00</td>
<td>2.33</td>
<td>3.29</td>
<td>1.83 to 4.25</td>
</tr>
</tbody>
</table>
The frequency distribution showed that most of the inbred progenies were in between the field emergence values displayed by either of the parents. However, a number of inbreds showed significantly higher value than the better parent PI610727 at both locations. This observation indicates that transgressive segregation for field emergence (during early season planting) exists in this population.

![Histogram for %field emergence at early season planting for RILs from Popn. 1(RTx430xPI567946). Data were obtained from 2008 season and were mean from two locations.](image)

**Figure 1.** Histogram for %field emergence at early season planting for RILs from Popn. 1(RTx430xPI567946). Data were obtained from 2008 season and were mean from two locations.

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**Genetic and QTL analysis for cold and optimal temperature germinability**

The linkage map constructed for the recombinant inbred population consists of 141 SSR markers and spans a total of 1005.1 cM in length (Figure 2). The map is composed of 10 linkage groups designated as Sbi 1 to Sbi10 based on chromosomal designation by Kim et al., 2005. In general, the positions of markers were consistent with previously reported map for sorghum which are populated by SSR markers (Bhatramakki et al., 2000; Menz et al, 2002; Wu and Huang, 2006; Salas-Fernandez et al., 2008). The order of markers in each linkage group was also projected to the whole
genome sequence of sorghum available at www.phytozome.net and was found to be fully supported by
the sequence data.

QTL for cold and optimal temperature germinability traits were represented as Germ12°C and
Germ30°C, respectively (Table 3, Figure 2). Analysis by composite interval mapping (CIM) revealed
two and three QTL each for cold and optimal germinability, respectively with significant LOD values
ranging from 2.3 to 9.0 (Table 3). Notably, Germ12-2.1 and Germ30-2.1 coincides and co localize
with each other with relatively high contribution to phenotypic variation (PVE) (Figure 2).

QTL analysis for early season field emergence and seedling vigor

Four QTL each were identified for field emergence from the USDA_LBK and ND_TAMU
locations, with LOD values ranging from 2.3 to 4.8. One QTL (Fearlygerm -9.3) was identified in
both locations which indicate its stability in influencing the expression of field emergence. However,
only one QTL for seedling vigor, (Fearlyvigor -4.1) was identified form this population (Table 3).

Table 3. Information on QTL identified from the recombinant inbred population through the
application of composite interval mapping (CIM).

<table>
<thead>
<tr>
<th>Trait</th>
<th>QTL name</th>
<th>Location</th>
<th>Chr</th>
<th>LOD Score</th>
<th>PVE(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Germ30°C</td>
<td>Germ30-1.1</td>
<td>-</td>
<td>1</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Germ30-1.2</td>
<td>-</td>
<td>1</td>
<td>3.0</td>
<td>7.21</td>
</tr>
<tr>
<td></td>
<td>Germ30-2.1</td>
<td>-</td>
<td>2</td>
<td>6.0</td>
<td>14.5</td>
</tr>
<tr>
<td>%Germ12°C</td>
<td>Germ12-2.1</td>
<td>-</td>
<td>2</td>
<td>9.0</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>Germ12-9.2</td>
<td>-</td>
<td>9</td>
<td>2.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Field emergence</td>
<td>Fearlygerm-1.2</td>
<td>USDA_LBK</td>
<td>1</td>
<td>2.3</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-7.1</td>
<td>USDA_LBK</td>
<td>7</td>
<td>2.8</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-9.2</td>
<td>USDA_LBK</td>
<td>9</td>
<td>4.8</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-9.3</td>
<td>USDA_LBK</td>
<td>9</td>
<td>3.8</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-1.1</td>
<td>ND_TAMU</td>
<td>1</td>
<td>2.8</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-4.1</td>
<td>ND_TAMU</td>
<td>4</td>
<td>3.8</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-9.1</td>
<td>ND_TAMU</td>
<td>9</td>
<td>3.8</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>Fearlygerm-9.3</td>
<td>ND_TAMU</td>
<td>9</td>
<td>2.4</td>
<td>8.8</td>
</tr>
<tr>
<td>Seedling vigor</td>
<td>Fearlyvigor-4.1</td>
<td>ND_TAMU</td>
<td>4</td>
<td>3.8</td>
<td>10.5</td>
</tr>
</tbody>
</table>
DISCUSSION

Tolerance to early season cool temperature is an important and desirable trait for major cereal crops like sorghum and rice. It is important especially for sorghum due to needs for excellent initial crop stand to ensure stable yield. Early season cold tolerance has also been proposed to aid sorghum plants in availing soil moisture from spring precipitation and the capacity to withstand cool soils associated with minimum tillage (Bacon et al., 1986). However, breeding for early season cold tolerance has been challenging, and previous study indicate that recurrent selection using adapted/elite population resulted only in a small improvement of the trait (Bacon et al., 1986). Thus, efforts to search for new sources of cold tolerance, followed by an effective and efficient incorporation into sorghum parental lines has received considerable attention (Yu and Tuinstra, 2000, Cisse and Ejeta, 2003; Franks et al., 2006).

Here, we analyzed a germplasm source of cold tolerance, PI610727, through its recombinant inbred progenies (F7 generation). A strong positive correlation between optimum and cold germinability was found among the inbred progenies, which indicates that vigorous germination is one of the important components of cold germinability. This strong relationship between the two traits has been shown for sorghum using a different population (Knoll et al., 2008a). Data from this study indicates that controlled test for germinability could serve as one of the indicator for cold tolerance to ensure that seeds are capable of germination, but that field emergence at early season planting had to be the main screening method to ensure efficient selection.

Recently, the application of DNA markers such as SSR followed by QTL analysis have been applied for studies of early season cold tolerance of sorghum (Knoll et al, 2008a; Knoll et al., 2008b). The use of QTL mapping has evolved to be an important tool for effective and efficient method of dissecting complex and polygenic agronomic traits in many crops, such as early season cold tolerance. Identification of tightly linked DNA markers to QTL regions can be used as efficient indicator for the
trait of interest and probably its appropriate allelic state through the technology of “marker assisted selection”.

A total of 14 QTL associated with four traits for cold tolerance were detected (Table 3 and Figure 2). The QTL were located in five sorghum chromosomes, namely 1, 2, 4, 7, and 9. Thirteen of these QTL are novel and had not been detected in previous reports by Knoll et al., 2008. In particular, it was revealed in this study that chromosome 9 harbors four QTL for field emergence. These QTL regions provide genetic support to the observation that cold germinability (under controlled condition) and optimum germination are important traits that contributes to field emergence in the early season planting. The QTL identified in this study were found in chromosomes with higher marker density except for regions in chromosome 4 and 7. These results suggest that mapping of more markers to Sbi3 to 8 are needed to fill in the gaps in these chromosomes. Thus, the number of QTL reported here could be considered as minimum for this population and that most of these QTL are those with large effects.

To summarize, QTL mapping for four traits associated with early season cold tolerance in sorghum was performed using an RIL population derived from a new source of cold tolerance. We also tagged the genome regions that have significant contributions to traits for early season cold tolerance. Identification of new QTL that exhibit stability across environments and those that control several correlated traits could provide new information on highly informative DNA markers for use in marker assisted selection that targets early season cold tolerance. The present study also provides additional phenotype marker association data for pyramiding of QTL responsible for cold tolerance in sorghum. However, it is proposed that further studies on QTL analysis for early season cold tolerance in sorghum should be continued especially through the development of maps with higher marker density for chromosomes 3 to 8, validation of phenotype across environments and evaluation of markers using other independent population such as that of Popn.2, BTx623xPI567946 RI population. Further study using heterogeneous inbred families or near isogenic lines of selected RILs from popn
I will provide useful information in cloning of actual genes involved. Hybridization of selected cold tolerant RILs to elite inbred parents are also of interest to study their performance in hybrid combinations.
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