Genetic Architecture of Chilling Tolerance in Sorghum Dissected with a Nested Association Mapping Population

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ABSTRACT Dissecting the genetic architecture of stress tolerance in crops is critical to understand and improve adaptation. In temperate climates, early planting of chilling-tolerant varieties could provide longer growing seasons and drought escape, but chilling tolerance (<15°C) is generally lacking in tropical-origin crops. Here we developed a nested association mapping (NAM) population to dissect the genetic architecture of early-season chilling tolerance in the tropical-origin cereal sorghum (Sorghum bicolor [L.] Moench). The NAM resource, developed from reference line BTx623 and three chilling-tolerant Chinese lines, is comprised of 771 recombinant inbred lines genotyped by sequencing at 43,320 single nucleotide polymorphisms. We phenotyped the NAM population for emergence, seedling vigor, and agronomic traits (75,000 data points from 16,000 plots) in multi-environment field trials in Kansas under natural chilling stress (sown 30–45 days early) and normal growing conditions. Joint linkage mapping with early-planted field phenotypes revealed an oligogenic architecture, with 5–10 chilling tolerance loci explaining 20–41% of variation. Surprisingly, several of the major chilling tolerance loci co-localize precisely with the classical grain tannin (Tan1 and Tan2) and dwarfing genes (Dw1 and Dw3) that were under strong directional selection in the US during the 20th century. These findings suggest that chilling sensitivity was inadvertently selected due to coinheritance with desired nontannin and dwarfing alleles. The characterization of genetic architecture with NAM reveals why past chilling tolerance breeding was stymied and provides a path for genomics-enabled breeding of chilling tolerance.

KEYWORDS Multiparental population Crop evolution Climate adaptation Cold tolerance Antagonistic pleiotropy Linkage drag

Adaptation to diverse environments has generated abundant genetic diversity in wild and domesticated plant species (Anderson et al. 2011; Meyer and Purugganan 2013). The genetic architecture of adaptation...
and/or growing season in temperate climates (Lyons 1973; Long and Spence 2013). Developing chilling-tolerant varieties could facilitate early planting to extend growing seasons, prevent soil moisture depletion, and shift growth and flowering to more favorable evapotranspirative conditions (Tuberosa 2012; Ma et al. 2015). For breeding chilling tolerance in tropical-origin crops, chilling-adapted germplasm from high-latitude zones and high-altitude tropical regions can be targeted as donors. Molecular mechanisms underlying cold tolerance (chilling and/or freezing temperatures) include C-repeat binding factor (CBF) regulon cold signaling (Thomashow 2001; Park et al. 2015; Wang et al. 2018), jasmonate signaling (Hu et al. 2013; Mao et al. 2019), and lipid remodelling (Li et al. 2004; Moelling et al. 2010).

Sorghum, a tropical-origin warm-season (C4) cereal, is among the major crops that are generally susceptible to chilling (Franks et al. 2006). Sorghum originated in tropical Africa (c. 5–10 thousand years ago) and diffused to temperate areas, including China (c. 800 years ago) and the United States (c. 200 years ago) (Kimber 2000). Diffusion of tropical sorghums to temperate climates has led to commercial sorghum industries covering several million hectares in US, Australia, Argentina, and China (Monk et al. 2014). Using a phytogeographic approach (Vavilov 1951), Chinese sorghum were targeted as chilling tolerance donors for conventional breeding starting in the 1960s (Stickler et al. 1962). However, characteristics of Chinese sorghums that are undesirable for US grain sorghum, particularly grain tannins and tall stature (>2 m) (Franks et al. 2006), have hampered breeding. Biparental linkage mapping identified chilling tolerance QTL tagged by the same molecular markers as grain tannins and plant height, but small populations and low marker density limited dissection of these traits (Knoll et al. 2008; Brown et al. 2008; Xiang 2009; Burrow et al. 2010; Wu et al. 2012). Several classical tannin (Tan1 and Tan2) and dwarfing (Dw1–Dw4) genes (Stephens 1946; Quinby and Karper 1954) have been cloned in recent years (Multani et al. 2003; Wu et al. 2012; Hilley et al. 2016, 2017), which could aid further trait dissection.

NAM populations can provide increased power for dissecting complex traits (Buckler et al. 2009; Ogut et al. 2015), particularly for adaptive traits where population structure confounds associations studies of natural populations (Bouchet et al. 2017). To dissect the genetic architecture of early-season chilling tolerance in sorghum, we developed and deployed a new nested association mapping (chilling NAM) resource. The chilling NAM population addresses a gap in existing sorghum NAM resources (Bouchet et al. 2017). Three chilling-tolerant Chinese founders, three chilling-tolerant Chinese founders and the chilling-susceptible reference line BTx623 (Paterson et al. 2009). We used the chilling NAM to dissect the genetic architecture of sorghum early-season chilling tolerance at a high resolution based on natural field stress conditions. This NAM study provides insights into the origin and persistence of chilling sensitivity in US grain sorghum, and reveals new strategies for genomics-enabled breeding in this system.

MATERIALS AND METHODS
Population development

The chilling NAM population consists of three biparental populations that share a common parent, the US reference line BTx623 (Paterson et al. 2009) (Figure S1). The NAM founders were selected based on their contrasting chilling responses from early planting in preliminary studies in Lubbock, Texas. Chilling-sensitive BTx623 was used as the seed parent in crosses with three chilling-tolerant Chinese founders, Niu Sheng Zui (NSZ; PI 568016), Hong Ke Zi (HKZ; PI 567946), and Kaoliang (Kao; PI 562744) in Lubbock, Texas. BTx623 is derived from Combine Kafir × SC170, an Ethiopian zerazeria caudatum (Menz et al. 2004). The resulting F1 progenies were self-pollinated to generate three segregating F2 populations. RILs were developed using single-seed descent by sowing to the F2 generation in Lubbock, Texas (summer nursery) and Guayanilla, Puerto Rico (winter nursery). The F6:7 RILs were derived by combining seeds of 3–4 uniform panicles. Additional seed increase of the NAM population was conducted in Puerto Vallarta, Mexico (winter nursery), by sowing the F6:7 plants to derive F8:8 RILs. Below, the Chinese founder name will be used when referring to a given RIL family (e.g., the NSZ family).

Early- and normal-planted field trials

Six early- and two normal-planted field trials were conducted in 2016, 2017, and 2018 in Kansas (Table S1). Three locations, two in eastern Kansas [Ashland Bottoms (AB), 39.14N -96.63W; Manhattan (MN), 39.21N -96.60W] and one in western Kansas [Agricultural Research, Hays (HA), 38.86N -99.33W], were used for field trials (Figure 1A). Abbreviated location name and the last two digits of the year (e.g., AB16 for Ashland Bottoms 2016) were assigned for each field trial. A suffix was added to the AB16 field trials, AB16_01 and AB16_02, as both were planted in AB with an interval of two weeks between plantings. The F6:7 RILs were planted in AB16, while F8:8 RILs were planted in AB, MN, and HA in 2017 and 2018. Each field trial contained two replicates of the NAM population. The NAM RILs were randomized within family in 2016, and completely randomized in 2017 and 2018 in each replicate block (Figures 1A and S2). Controls in each field trial comprised chilling-tolerant Chinese accessions NSZ, HKZ, Kao, and Shan Qui Red (SQR; PI 656025), chilling-sensitive inbreds BTx623 and RTx430, and US commercial grain sorghum hybrid Pioneer 84G62.

Five early-planted (EP, natural chilling stress) trials were sown in April and one in early May (MN17), 30–45 days earlier than normal sorghum planting in Kansas (Grain Sorghum Production Handbook 1998). The EP trials, except MN17, experienced chilling stress (<15°C) during emergence (Table S1 and Figure S3). Optimal temperatures (>15°C) prevailed in MN17 during emergence, but one-week-old seedlings experienced chilling stress (5–13°C). Normal-planted (NP, optimal temperature) field trial was sown in June when the soil temperatures were optimal for sorghum cultivation (>15°C). AB18 was considered as the second NP trial, although planted in early May, as optimal conditions prevailed during emergence and seedling growth.

Field phenotyping

Seeding phenotypes of the NAM population were evaluated under early- and normal-planted field trials. Prefixes EP and NP were included for each seedling trait to differentiate phenotypes from early- and normal-planted trials, respectively. Emergence count (EC) was scored on a scale of 1–5 that represented 20, 40, 60, 80, and 100% emergence, respectively. Three seedling vigor (SV) ratings (SV1–SV3), scored independent of emergence count, were collected at week-1, -2, and -4, respectively, after emergence. SV was scored on a rating scale of 1–5 with a rating of 1 and 5 for low and robust vigor, respectively (Figure 1B). A previously described SV scale (Maiti et al. 1981) was modified (1 for high and 5 for low SV) for consistency with EC rating. Repeatability of SV rating, SV2 (AB17) and SV3 (MN17), was tested with SV ratings collected simultaneously by different individuals. Early-planted damage rating (EPDR), based on visual damage observed two days after a severe chilling stress event, was scored on a 1–5 rating scale representing seedling death/severe leaf-tip burning, leaf-tip burning, severe chlorosis, mild/partial chlorosis, and no chilling damage.

5406 | S. R. Marla et al.
symptoms, respectively. Seedling height was measured manually one month after initial emergence in each location.

Plant height and flowering time (days to flowering after emergence), the major agronomic traits, were collected from three (AB16_b1, MN17, and MN18) and two (AB16_b1 and MN17) field trials, respectively. Agronomic suitability of the NAM population as US grain sorghum, which included semi-dwarf stature, panicle exertion, standability, and compact panicle architecture, was screened in AB16_b1. Presence or absence of grain tannins in field-grown samples (from Puerto Vallarta, Mexico) of each RIL was determined using bleach test with SQR, a Chinese accession containing grain tannin, as a positive control (Wu et al. 2012). Fifteen seeds from each RIL were transferred into a 2 ml tube and 1 ml bleach solution (3.5% sodium hypochlorite and 5% sodium hydroxide) was added. RILs with tannins turned black after 30 min and were scored as 1. By contrast, nontannin RIL seed did not change their color and were scored as 0.

### Statistical analysis of phenotypes

Trait correlation between locations was determined using the averaged seedling trait ratings of two replicates from each field trial. Pearson pairwise correlation analysis was performed using pairs.panels function in psych R package. Broad sense heritability ($H^2$) estimate of EP and NP field phenotypes was calculated with seedling ratings from six EP and two NP field trials, respectively. Seedling traits $H^2$ was calculated from variance components generated with the lmer (Bates et al. 2015) R package as described earlier (Boyles et al. 2017). All components were treated as random effects and replicates were nested in location-by-year interaction:

$$\text{lmer(Trait} = (1 \mid G) + (1 \mid L) + (1 \mid Y) + (1 \mid R\%in\%L:Y) + (1 \mid G:L) + (1 \mid G:Y)$$

and broad-sense heritability was calculated using the equation:

$$H^2 = \frac{\sigma^2_G}{\sigma^2_G + \frac{\sigma^2_G\text{loc}}{L} + \frac{\sigma^2_G\text{year}}{Y} + \frac{\sigma^2_E}{IT}}$$

where $G$ is the genotype, $L$ is the location, $Y$ is the year, $R$ is the replicate, and $E$ is the error term. Environment main effects were not included in the denominator as they do not influence response
to selection (Holland et al. 2003). Best linear unbiased predictions (BLUPs) of EP and NP seedling traits were generated using the model for estimating \( H^2 \).

**DNA extraction and genotyping**

Genotyping-by-sequencing (GBS) was conducted on one-week-old seedlings of the F\(_{R7}\) RILs and the four NAM founders (Figure S1). Leaf tissue (\( \sim 50 \) mg, pooled from three seedlings) from each RIL was transferred into a 96-deepwell plate, lyophilized, and stored at -80\(^\circ\)C. One ball bearing was added to each well and the leaf tissue was ground with a Retsch Mixer Mill MM400 tissue grinder (Vernon Hills, IL, USA). Genomic DNA was extracted using Qiagen BioSprint 96 DNA plant kit (Germantown, MD, USA). DNA was quantified with Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Grand Island, NY, USA) using Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) at the Kansas State University Integrated Genomics Facility. Each sample was normalized to contain 10 ng/\( \mu \)l DNA using QIAexpress Liquid Handling System (Germantown, MD, USA). Six \( \mu \)l of DNA was transferred to a 96-well PCR plate and adapters were added. ApeKI enzyme was used for restriction digestion and GBS libraries were prepared as described previously (Elshire et al. 2011; Morris et al. 2013a). Illumina HiSeq 2500 Rapid v2 sequencing system was used for 100-cycle single-end sequencing of two 384-multiplexed libraries at the University of Kansas Medical Center Genome Sequencing Facility.

GBS data from the chilling NAM resource was combined with previously published ApeKI GBS data from \( \sim 10,323 \) diverse accessions (Hu et al. 2019), aligned to the BTx623 reference genome v3.1 (McCormick et al. 2018), and SNP calling was performed using Tassel 5.0 GBS v2 pipeline (Glaubitz et al. 2014). GBS of the chilling NAM population provided 528,065 single nucleotide polymorphisms (SNPs) (Figure S1). GBS data were filtered for 80% missingness (PM) and 0.05 minor allele frequency (MAF, based on the chilling NAM population that contains 50% BTx623 alleles and \( \sim 16 \)% alleles from each Chinese parent). 61,428 SNPs were retained and these SNPs were separated by individual chromosomes and imputed using Beagle 4.1 (Browning and Browning 2013). Additional filtering for markers and RILs with \( \geq 15\% \) residual heterozygosity retained 43,320 SNPs and 750 RILs for joint linkage mapping.

**Population genetic analyses**

Genetic structure of the chilling NAM population was characterized with respect to global sorghum germplasm. First, the chilling NAM and global accessions GBS data were filtered for 80 PM and 0.01 MAF (to avoid discarding rare alleles), and the retained SNPs (265K) were imputed using Beagle 4.1 (Browning and Browning 2013). Next, two PCA axes were built with previously published ApeKI GBS data of 401 global sorghum accessions (Morris et al. 2013a), and chilling NAM founders and RILs were projected on these axes. Principal component analysis (PCA) of global germplasm was performed using prcomp function in R. Coordinates for the chilling NAM population were calculated with the predict function in R.

Neighbor-joining analysis, using TASSEL 5.0 (Glaubitz et al. 2014), was conducted with 61,428 SNPs to characterize the genetic relatedness of the chilling NAM population. In TASSEL, modified Euclidean distance was used as distance model and neighbor-joining as the tree algorithm. A neighbor-joining tree was constructed with Ape package (Paradis et al. 2004) in R (R Core Team 2018). SNP density was calculated with VCFTools (Daneczek et al. 2011) in 200kb windows. Linkage disequilibrium (LD) decay was estimated, using pairwise comparisons of \( \sim 55–70K \) GBS SNPs, individually for the three NAM families with PopLDdecay v.3.29 package (Zhang et al. 2018). LD decay of 176 Ethiopian and 29 Chinese landraces (genotyped previously with ApeKI) (Lasky et al. 2015) was estimated for comparison. Ethiopian and Chinese germplasm LD decay was calculated using \( \sim 100K \) and \( \sim 57K \) SNPs, respectively. Parameters were set for -MaxDist as 500 kb and -MAF as 0.05. LD decay curves were plotted based on \( r^2 \) and the distance between pairs of SNPs.

**Linkage mapping analysis**

The NAM founders genotypes were used for constructing genetic linkage maps with the R/qtl package (Broman et al. 2003). The level of heterozygosity between the parental lines was determined using VCFTools–net function (Danecek et al. 2011). The NAM founders were filtered for 20 PM and \( < 0.4 \) MAF and the retained SNPs were used to retrieve the NAM population genotypes from the GBS dataset. SNP imputation was conducted for each family separately using Beagle 4.1 (Browning and Browning 2016). RILs with \( > 85\% \) missing data or \( > 80 \) crossovers were dropped. Duplicate markers (i.e., mapping to the same location; \( \sim 200 \) from each family) were identified using the findDupMarkers function and dropped with drop.markers based on the R/qtl tutorial recommendation. Genetic linkage maps for each NAM family were generated using the Haldane function. The Droporemark function in R/qtl was used to discard problematic markers that increase chromosome length. Genetic linkage maps were reconstructed for each NAM family. Composite interval mapping (CIM) (Zeng 1994), with R/qtl, was used for performing linkage mapping and significant QTL were determined based on the threshold level defined by computing 1000 permutations. Allelic effects were defined as positive or negative effects of the BTx623 allele. LOD support interval for individual QTL was obtained with the lodint R/qtl function. CIM was performed with plant height, flowering time, and grain tannin data to validate the generated genetic linkage maps. BLUPS of seedling traits, EC and SV1–3, from early- and normal-planted field trials were used for CIM. Additionally, linkage mapping was performed for individual field trials with the averaged data of two replicates from each location.

**Joint linkage mapping**

Joint linkage mapping (JLM) was conducted with 43,320 GBS SNPs and seedling trait BLUPs from 750 RILs. In addition, JLM was performed individually for each location with the averaged data of two replicates. Mapping power and resolution of the chilling NAM population was validated using plant height, flowering time, and grain tannin data. Stepwise regression approach in TASSEL 5.0 (Glaubitz et al. 2014), which uses forward inclusion and backward elimination stepwise method, was used to perform JLM. Entry and exit limit of the forward and backward stepwise regressions was 0.001 and threshold cut off was set based on 1000 permutations. JLM was performed using the following equation:

\[
y = b_0 + \alpha f + \sum_{i=1}^{k} x_i b_i + e_i
\]

where \( b_0 \) is the intercept, \( \alpha f \) is the effect of the family of founder line \( f \) obtained in the cross with the common parent (BTx623), \( \alpha \) is the coefficient matrix relating \( f \) to \( y \), \( b_i \) is the effect of the \( i \)th identified locus in the model, \( x_i \) is the incidence vector that relates \( b_i \) to \( y \) and \( k \) is the number of significant QTL in the final model. Allelic effect for each QTL was expressed relative to the BTx623 allele, where alleles with positive- or negative-additive effects were derived from BTx623 or Chinese founders, respectively. Based on the average genome-wide recombination rate of 2.0 cM/Mb for sorghum...
(Mace et al. 2009; Bouchet et al. 2017), QTL for one or more seedling traits that mapped within a 2 Mb interval were assigned a common name. For example, qSbCT04.62 to describe QTL detected on chromosome 4 close to 62 Mb.

**Sequence variant analysis**

CBF and Tan1 genes, colocalizing with chilling tolerance QTL, were used for sequence variant analysis. Two overlapping primer pairs were used to amplify these genes from the Chinese founders (primer sequences are included in Table S2). 50% glycerol and 25mM MgCl2 were added to the master mix for stabilizing the PCR reaction. PCR product purification and Sanger sequencing were performed at GENEWIZ (South Plainfield, NJ). Clustal Omega and Expasy translate were used for sequence alignment and predicting the peptide sequences of CBF1 and Tan1 genes.

**Ecophysiological crop modeling**

CERES-Sorghum crop model (White et al. 2015) in the Decision Support Systems for Agro-technology Transfer-Crop Simulation Model software (Jones et al. 2003) was used to predict the value of early planting for grain sorghum in the Kansas production environment. This model simulates daily physiological processes using a base temperature of 8°C (White et al. 2015) and has effectively predicted sorghum grain yield in Kansas (Staggenborg and Vanderlip 2005; Araya et al. 2018). We consider that this model assumes chilling tolerance by default, since it does not model damage due to chilling temperatures. A full-season (late-maturing) photoperiod insensitive grain sorghum hybrid, used in previous crop modeling, was used in this study (Araya et al. 2018). Simulations were performed under rainfall conditions at four representative Kansas locations, Colby (39.39N, -101.06W), Garden City (37.99N, -101.81W), Hays (38.84N, -99.34W), and Manhattan (39.20N, -96.55W), from a 30 year period (1986-2015). Historical weather data for each of these locations was obtained from Kansas Mesonet (2019). Simulations were started on January 1 to account for the effect of precipitation on soil moisture and the onset of soil evaporation. Early (April 15), normal (May 15), and late (June 15) planting scenarios were simulated and (i) available precipitation, (ii) days of water stress after anthesis, and (iii) final grain yield were analyzed.

**Data availability**

Sequencing data are available in the NCBI Sequence Read Archive under project accession SRP8838986. Figure S1 depicts the experimental design for mapping early-season chilling tolerance. Figure S2 contains the field layout of trials. Figure S3 shows daily minimum air temperature across field trials. Figure S4 contains seedling traits of US and Chinese founders. Figure S5 includes SNP density map and genetic structure of the chilling NAM population. Figures S6 and S7 show trait correlation across trials. Figure S8 visualizes genetic linkage maps for each family. Figure S9-S12 are comparisons of EP and NP JLM for emergence count (EC), seedling vigor (SV1, SV2, and SV3). Figures S13-18 are JLM from individual field trials. Figures S19 and S20 have CIM of plant height and grain tannins, respectively. Figure S21 has JLM of plant height and flowering time, and Figure S22 has JLM with grain tannins. Figure S23 has JLM QTL for chilling tolerance in tannin vs. nontannin RILs. Figure S24 describes ecophysiological crop simulations of early-season chilling tolerance. File S1 contains genetic linkage maps. File S2 contains Hapmap data. File S3 contains R scripts used for analyses and seedling trait BLUPs. Table S1 has phenotyping details for each field trial. Table S2 has primers used to amplify CBF and Tan1 genes. Tables S3 and S4 contain CIM QTL from EP- and NP seedling trait BLUPs, respectively. Tables S5 and S6 have JLM QTL from NP seedling trait BLUPs and agronomic traits, respectively. Table S7 is a comparison of CIM QTL with previously-mapped chilling tolerance QTL. Table S8 has EP seedling trait ratings of RILs selected for favorable plant height. The chilling NAM population seeds will be submitted to the USDA National Plant Germplasm System (https://www.ars-grin.gov/). Please contact G.B. (gloria.burow@ars.usda.gov) or the corresponding author for availability. Supplemental material available at figshare: https://doi.org/10.105387/g3.9755336.

**RESULTS**

**Development of NAM population for chilling tolerance studies**

The chilling NAM population was generated from crosses of a US reference line BTx623 with three Chinese lines, NSZ, Kao, and HKZ (Figure S1). The resulting chilling NAM population (n = 771) comprised 293, 256, and 222 RILs for the NSZ, Kao, and HKZ families, respectively. Our chilling tolerance studies of the NAM founders and RILs were based on natural chilling events in field trials sown 30-45 days earlier than normal. In early-planted field trial (Figures 1A–B) the Chinese founders had significantly greater emergence and seedling vigor (P < 0.05) than BTx623 (Figures 1C, 1D, and S4). By contrast, no difference was observed in emergence (NPEC) between the founders under normal-planting (Figure S4). Chinese founder lines were much taller (~3 m) at maturity than BTx623 (1.2 m) (Figure 1E), but little variation was observed for flowering time among the founder lines (4.5–5.3; P < 0.05; Figure 1F). Grain tannins were present in the Chinese accessions and absent in BTx623 (Figure 1G).

**Genetic properties of the chilling NAM population**

The filtered GBs data set for the chilling NAM population comprised genotypes at 43,320 SNPs. SNP densities were higher in telomeres than pericentromeric regions (Figure S5A). To check the population’s quality and understand its genetic structure, NAM RILs and founders were projected onto PCA axes built from a global sorghum diversity panel (Figure 2A), which reflect geographic origin and botanical race (Harlan and de Wet 1972; Morris et al. 2013a). As expected, the Chinese founders clustered with dura sorghums of Asia and East Africa, while BTx623 was positioned midway between kafir and caudatum clusters, consistent with its pedigree (Menz et al. 2004) (Figure 2A). The three half-sib families of the chilling NAM population were clustered together, midway between the Chinese founders and BTx623. NJ analysis (Figure S5B) and PCA (Figure S5C) of the chilling NAM population by itself confirmed the expected family structure for NSZ and Kao, with each family forming a single cluster. Two clusters were observed for the HKZ family. We assigned HKZ RILs into HKZa (nREL = 121) or HKZb (nREL = 101) subfamilies, with the HKZb subfamily representing the cluster with PC1 > 40 (and the longer branch on NJ dendrogram). The LD rate decay (to genome-wide background) was slower in NAM families (~500 kb) compared to diverse accessions from China and Ethiopia (~20 kb) (Figure 2B).

**Repeatability and heritability of field phenotypes**

RILs were scored for emergence and seedling vigor under early- and normal-planted field trials. Early (EPSV1) and later (EPSV2, EPSV3) seedling vigor ratings were strongly correlated (0.7–0.8), as were ratings made by different individuals on the same day (0.7–0.8) (Figure S6). By contrast, the correlation across RILs between early- and normal-planted seedling traits was low (0.1–0.3). Broad sense heritability (H2) across locations and years for early-planted seedling traits was intermediate (0.4–0.5) (Table 1), while H2 was higher (0.5–0.8) for
seeding traits from normal-planted field trials. $H^2$ for seedling height (in early-planted field trials) was close to zero (0.03), while plant height at maturity was highly heritable (0.9). Based on the averaged data of two replicates within each field trial, low to intermediate correlation (0.1–0.4) was observed with the same seedling trait among locations for early-planted trials (Figure S7).

**Composite interval mapping of early-season chilling tolerance**

Genetic linkage maps were constructed for each family (NSZ: 1341 markers, 257 RILs; Kao: 1043 markers, 219 RILs; HKZa: 1150 markers, 107 RILs) (Figure S8). Map lengths were similar for the NSZ, Kao, and HKZa families (1403 cM, 1381 cM, and 1295 cM, respectively) and individual RILs contained 2–4 crossovers. To map putative chilling tolerance loci, composite interval mapping (CIM) was first conducted in individual families using 1000–1300 markers and early-planted seedling trait BLUPs (EPEC, EPSV1–3). CIM detected 6–8 QTL, which explained 16–28%, 8–23%, and 12–36% of variation for early-planted seedling traits in the HKZa, Kao, and NSZ families, respectively (Table S3). The QTL on chromosome 4 was detected in all NAM families, with the positive allele inherited from the Chinese founder in each case. CIM of normal-planted seedling BLUPs (NPEC and NPSV1–NPSV3) identified 4–9 QTL contributing to emergence and SV in the HKZa, Kao, and NSZ families, respectively. Few overlaps were observed among QTL detected for early- and normal-planted seedling traits (Tables S3 and S4). As chilling stress varied among locations (Figure S3), QTL mapping was conducted for each field trial separately to check the stability of QTL across locations. The QTL on chromosomes 4 and 7 were detected across families in four and two early-planted trials, respectively.

**Joint linkage mapping of early-season chilling tolerance**

To leverage data across families, JLM was performed with 43,320 SNPs and field phenotypes from 750 RILs (including the HKZb family) (Figure 3A–E). JLM of seedling trait BLUPs (derived from ~12,000 early-planted plots) identified 15 QTL, seven of which were detected for multiple seedling traits (Figure 3D and Table 2). Each QTL explained 1–9% of phenotypic variation. In total, the QTL explained 21–41% variation for emergence and seedling vigor. Positive alleles were inherited from the Chinese founders, except for the allele at chromosome 3. The QTL on chromosomes 2 and 4 were detected for every early-planted seedling trait. The chromosome 1 and 5 QTL were detected with all seedling vigor traits, while chromosome 7 and 9 were mapped with two early-planted seedling traits (Figure 3D). The QTL on chromosomes 2 and 4 colocalized (<1 Mb) with classical tannin genes, Tan2 and Tan1 (Wu et al. 2012; Morris et al. 2013b), and chromosomes 7 and 9 loci colocalized with classical dwarfing genes, Dw3 and Dw1 (Multani et al. 2003; Hilley et al. 2016).

To check the stability of QTL across locations and years, JLM was performed separately by location. The QTL on chromosome 9 was detected in three early-planted locations, while QTL on chromosomes 2 and 7 were mapped in two locations (Figure 3B and S13–S18). The chromosome 4 QTL was consistently detected across early-planted field locations.
locations and years. The only exception was the MN17 field trial, which emerged under optimal conditions and experienced chilling one week later, where the chromosome 4 QTL was not detected (Figures 3B and S16). Among the loci detected with JLM of field phenotypes from early- and normal-planted individual field trials (Figures 3A–B), few overlaps were observed.

The most significant and consistent QTL (qSbCT04.62; Table 2) colocalized with CBF gene Sobic.004G283201 (120 kb from the peak SNP), ortholog of the canonical Arabidopsis cold acclimation regulator CBF1 (Thomashow 2001; Park et al. 2015). However, sequencing of the CBF gene from the Chinese founders revealed no change in their predicted peptide relative to the BTx623 reference sequence.

**Mapping for agronomic traits and grain tannin**

CIM and JLM was conducted to identify loci underlying plant height, flowering time, and grain tannins. CIM detected three plant height QTL in the HKZa family (Table S6 and Figure S19), and two each in the NSZ and Kao families, explaining 30–82% of plant height variation. Two plant height QTL detected on chromosomes 7 and 9, colocalized with classical dwarfing genes Dw3 and Dw1, respectively (Multani et al. 2003; Hilley et al. 2016). JLM identified six plant height QTL, of which alleles at four and two QTL contained negative and positive effects, respectively (Figures 3C and S21, and Table 3). Three QTL of major effect explained 85% plant height variation. Major height loci were 12 kb and 0.1 Mb from Dw3 and Dw1 genes, respectively.

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**Figure 3** Joint linkage mapping (JLM) of chilling tolerance and undesirable traits JLM of seedling traits from individual (A) normal (NP) and (B) early planting (EP) field trials. Field location and year were included as prefixes for each seedling trait. Five NP traits that failed to detect QTL were excluded from the figure but used for calculating NP seedling trait BLUPs. JLM with seedling trait BLUPs, generated with ~75,000 data points from ~16,000 field plots, from (C) normal and (D) early planting. Additionally, (E) JLM of plant height, flowering time, and grain tannins were included. Classical dwarfing and tannin genes were noted with gray dashed lines. Chilling tolerance QTL detected under early planting are noted with blue dashed lines and green lines noted chilling tolerance QTL detected under both early and normal planting. The QTL under normal planting were noted with red dashed lines. Positive or negative effects of the BTx623 allele was indicated in orange or blue colors, respectively. The percentage of variation explained is proportional to the width of the box for each locus and loci explaining phenotypic variation >10% are noted with circles. Abbreviations: EC, emergence count; SV1–3, seedling vigor1–3; CD, chilling damage.
Two tannin loci, one mapped to chromosome 4 from an earlier reported Tan2 candidate gene (Wu et al. 2012; Morris et al. 2013b) (Figure S22, and Table 3).

**DISCUSSION**

**A NAM resource to dissect the genetic architecture of chilling tolerance**

Characterizing the genetic architecture of adaptive traits provides insight into mechanisms of adaptation (Orr 2005) and guides strategies for breeding (Bernardo 2008). The NAM approach has been used to increase power and accuracy for dissection of complex adaptive traits in several widely adapted crop species (Buckler et al. 2009; Nice et al. 2016; Bouchet et al. 2017). By using temperate-adapted founders with contrasting chilling responses (Figures 1C, 1D, and S4), the chilling NAM resource addresses a gap in available sorghum NAM resources (Bouchet et al. 2017). Together, the chilling NAM and global NAM populations (Bouchet et al. 2017) make up a resource of >3000 lines for complex trait dissection in sorghum. Given the founder lines originated from different botanical races (kafr-caudatum vs. durra; Figure 2A), the chilling NAM population should harbor abundant diversity for future studies of adaptive traits. Anecdotal field observations suggest the population harbors variation in vegetative pigmentation, disease susceptibility, and panicle and stem architecture.

The quality of the chilling NAM resource (i.e., RILs and corresponding SNP genotypes) developed in our study is validated by the precise mapping (<100 kb) of cloned dwarfs (Dw1 and Dw3) and tannin (Tan1) genes (Figure 3, Table 3). Similarly, several major QTL (qSbCT04.62, qSbCT02.08, qSbCT07.59, and qSbCT07.59) were encompassed within the QTL intervals detected previously (Knoll et al. 2008; Burrow et al. 2010) (Table S7). Notably, however, the greater population size (~4–fivefold) and marker density (>100-fold) with NAM relative to earlier studies greatly improved the mapping resolution (>10-fold; Table S7) and power (i.e., several additional loci identified). Family structure and LD decay of the chilling NAM population generally matches expectations based on population design and observations from previous NAM populations (Bouchet et al. 2017). Genotypic similarity of HKZa and HKZb RILs make up a resource of 3000 lines for complex trait dissection in sorghum. Given the founder lines originated from different botanical races (kafr-caudatum vs. durra; Figure 2A), the chilling NAM population should harbor abundant diversity for future studies of adaptive traits. Anecdotal field observations suggest the population harbors variation in vegetative pigmentation, disease susceptibility, and panicle and stem architecture.

Although flowering time varied little among the founders (Figure 1E), transgressive segregation enabled detection of seven flowering time loci (four, two, and one QTL in the NSZ, Kao, and HKZa families, respectively) which explain 20–28% of variation (Table S6). JLM with flowering time detected 10 QTL that explained 33% variation (Figures 3C and S21, and Table 3), three of which co-localized with previously identified flowering time/maturity genes, TOC1/BN2, ma1, and CN8. CIM of grain tannin presence/absence identified a major QTL on chromosome 4 in each family, with the Chinese parent conferring tannin presence in all cases (Figure S20). The locus colocalizing with Tan1 explained 77, 34, and 100% of grain tannin variation in the HKZa, NSZ, and Kao families, respectively (Table S6). JLM identified two tannin loci, one mapped ~70 kb from Tan1 and the other mapped ~1.4 Mb from an earlier reported Tan2 candidate gene (Wu et al. 2012; Morris et al. 2013b) (Figure S22, and Table 3).
regarding the pedigree of HKZb RILs does not diminish their usefulness as a part of the NAM resource (e.g., Figure 3).

QTL mapping from multi-environment trials clearly identified a major oligogenic component of chilling tolerance (Figure 3), consistent with previous work (Knoll et al. 2008; Burow et al. 2010; Fiedler et al. 2016; Ortiz et al. 2017). In keeping with the breeding goals, we considered all QTL that controlled performance under chilling stress (emergence, seedling vigor, or both) as chilling tolerance loci (Table 2), regardless of whether they also controlled performance under normal conditions. As chilling tolerance trials were conducted in a field environment, heritability and QTL effect sizes (Tables 1 and 2) were somewhat reduced compared to previous experiments under controlled conditions (Knoll et al. 2008). While replicability of field phenotyping for abiotic stress is a major challenge (Araus and Cairns 2014), observing plant performance under field conditions may increase the likelihood that genetic discoveries will translate to farmer fields (Cobb et al. 2018). A common limitation for molecular breeding of stress tolerance has been a lack of QTL stability (i.e., QTL × environment interaction) (Bernardo 2008). The overlapping of multi-environment chilling tolerance QTL from this study with QTL previously identified in the fields in Texas and Indiana (Table S7) provides evidence of their stability across a wide range of early-season chilling scenarios.

The genetic basis of early-season chilling tolerance

Molecular networks for cold sensing and response appear to be largely conserved across plants (Knight and Knight 2012; Dong et al. 2019). These findings are consistent with long-standing observations of homologous variation in cold tolerance across diverse grasses, including sorghum (Vavilov 1951). For this reason, we considered whether NAM provides evidence that chilling tolerance in Chinese sorghum is due to derived variation at canonical cold tolerance genes (e.g., CBFs, COLD1, SENSITIVE TO FREEZING2, etc). Overall, we found little evidence that the chilling tolerance in Chinese sorghum is due to variation in canonical cold regulators (i.e., little localization between QTL and sorghum orthologs of known plant cold tolerance genes). For instance, the CBF gene near the chilling tolerance QTL on chromosome 4 shows no coding sequence differences among the founder lines and a previous study showed no chilling-responsive expression of this CBF in chilling-tolerant NSZ (Marla et al. 2017). These findings suggest that a different closely linked gene, or the nearby Tan1 gene, underlie this chilling tolerance QTL. No other QTL colocalized with orthologs of known plant cold tolerance genes (Thomashow 2001; Welti et al. 2002; Moellering et al. 2010).

The chilling tolerance QTL observed in our study may represent novel chilling tolerance mechanisms in sorghum, or conserved mechanisms not yet described in model plants. Fine-mapping and positional cloning of each chilling tolerance QTL (Ma et al. 2015) will be needed to address these or other hypotheses on the molecular basis of chilling tolerance in sorghum. Still, the genetic architecture provides some potential clues. Surprisingly, chilling tolerance QTL colocalized closely with classical tannin (Tan1 and Tan2) and dwarving genes (Dw1 and Dw3) (Figure 3), four of the five most important genes under selection by US sorghum breeders in the 20th century (the fifth important gene, not colocated with chilling tolerance QTL is Maturity1) (Karper and Quinby 1946; Stephens et al. 1967; Wu et al. 2012; Morris et al. 2013a). This finding contradicted our original hypothesis of weak coupling-phase linkage of chilling susceptibility alleles with nontannin and dwarving alleles. The colocalization itself could be due to (i) tight linkage (e.g., <1 Mb) of chilling tolerance loci to classical tannin and dwarving loci or (ii) pleiotropic effects of classical tannin and dwarving loci on chilling tolerance.

First we considered whether coinheritance of tannin and chilling tolerance alleles could be due to a pleiotropic effect of seed pigmentation regulators (Tan1 and Tan2) on chilling tolerance. Although BTx623 and Chinese parents all harbor functional Tan2 alleles (based on complementary dominance of tannin genes), the chilling tolerance QTL near Tan2 could be due to allelic variation at Tan2 that does not alter grain tannins. A conserved MBW ternary complex controls

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**Table 3: Joint linkage mapping of plant height, flowering time, and grain tannins**

| Trait  | QTL          | QTL SNP     | PVEb | Additive effectc | Known locid | Distance to known loci |
|--------|--------------|-------------|------|------------------|-------------|------------------------|
| PHT    | qSbPHT_7-59  | S7_59675001 | 32   | −21              | Dw3         | 0.1 Mb                 |
|        | qSbPHT_9-57  | S9_57051085 | 20   | −17              | Dw1         | 12 kb                  |
|        | qSbPHT_1-67  | S1_67896587 | 0.5  | 0.7              |             |                        |
|        | qSbPHT_2-47  | S2_47291440 | 2    | −5               |             |                        |
|        | qSbPHT_7-59  | S7_59956049 | 33   | −21              | Dw3         | 0.1 Mb                 |
|        | qSbPHT_1-63  | S1_63253487 | 1    | 7                |             |                        |
| FT     | qSbFT_9-58   | S9_58468999 | 8    | −1.5             | CN8         | 3.5 Mb                 |
|        | qSbFT_2-64   | S2_6321883  | 6    | 1.4              |             |                        |
|        | qSbFT_8-59   | S8_59740114 | 2    | 0.9              |             |                        |
|        | qSbFT_1-56   | S1_56436041 | 3    | 1                |             |                        |
|        | qSbFT_3-01   | S3_1441099  | 3.03 | −0.96            |             |                        |
|        | qSbFT_4-63   | S4_4355602  | 2.01 | 0.82             | CN2         | 8.6 Mb                 |
|        | qSbFT_4-54   | S4_4231126  | 3.11 | −1.32            |             |                        |
|        | qSbFT_1-14   | S1_41862315 | 1.97 | 0.92             |             |                        |
|        | qSbFT_10-56  | S10_56045853| 0.69 | −0.4             |             |                        |
|        | qSbFT_6-40   | S6_40299229 | 2.55 | −0.89            | Ma1         | 5 kb                   |
| Tannin | qSbTan_4-62  | S4_62389178 | 72   | −0.4             | Tan1        | 73 kb                  |
|        | qSbTan_4-62  | S4_62261292 | 46   | −0.4             | Tan1        | 54 kb                  |
|        | qSbTan_4-61  | S4_61963687 | 22   | −0.3             | Tan1        | 0.3 Mb                 |
|        | qSbTan_2-09  | S2_9390193  | 0.06 | 0.02             | Tan2        | 1.4 Mb                 |
|        | qSbTan_10-59 | S10_5959345 | 2    | 0.2              |             |                        |

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a Plant height (PHT), flowering time (FT), and grain tannin phenotypes were used for JLM.
b Percentage of variation explained (PVE).
c Positive or negative effects of the BTx623 allele.
d Previously characterized PHT, FT, and grain tannin genes colocalizing with the mapped QTL.
sensitivity of US sorghums. Coinheritance of chilling tolerance loci with wildtype alleles of classical dwarfs (Dw1 and Dw3) and tannin (Tan1 and Tan2) genes suggest tropical-origin sorghums are chilling-tolerant. Inadvertent selection of chilling-sensitive alleles with favorable dwarfs (Dw1 and Dw3) and nontannin (Tan1 and Tan2) alleles resulted in persistence of chilling sensitivity in US sorghums, despite breeding for chilling tolerance over the past 50 years.

biosynthesis of flavonoids and tannins in plants via interactions of Myb and bHLH transcription factors with a WD40 transcriptional regulator (Nesi et al. 2000; Gu et al. 2011; Gao et al. 2018). Among sorghum tannin genes, Tan1 encodes the WD40 component (Wu et al. 2012) and Tan2 colocalizes with the bHLH transcription factor (Sobic.002G076600) (Morris et al. 2013b) orthologous to Arabidopsis TRANSPARENT TESTA8 (AtTT8) and rice red grain gene (OrRe) (Nesi et al. 2000; Gu et al. 2011). The MBW complex has pleiotropic effects on abscisic acid-mediated seed dormancy and polyphenol-mediated protection from soil-borne pathogens (Helsper et al. 1994; Gu et al. 2011; Jia et al. 2012), which could contribute to emergence and seedling vigor under chilling. The chilling tolerance QTL qSbCT02.08 detected in JLM of nontannin RILs (Figure S23) suggests that early-season chilling tolerance does not require seed tannins, even if the trait is under the control of the MBW complex. The existence of a Chinese accession Gai Gaoliang (PI 610727) that is chilling-tolerant but lacks grain tannins (Burow et al. 2010) supports this hypothesis.

Next we considered whether plant height alleles (Dw1 and Dw3) could have pleiotropic effects on chilling tolerance that explain their colocalization with qSbCT07.59 and qSbCT09.57 (Figure S9). Dw1, which colocalized with qSbCT09.57, encodes a novel component of brassinosteroid (BR) signaling (Hirano et al. 2017). BR signaling controls cold tolerance mechanisms in tomato (Xia et al. 2018) and Arabidopsis (Eremina et al. 2016) so colocalization of qSbCT09.57 with Dw1 could reflect a pleiotropic chilling tolerance effect of DW1 BR signaling. Dw3, which colocalized with qSbCT07.59, encodes an auxin transporter. However, to our knowledge, no reports have demonstrated a role of auxin signaling in chilling tolerance.

Origins and consequences of the genetic architecture of chilling tolerance

Chilling sensitivity of US sorghum has generally been understood to be a result of sorghum’s tropical origin (Stickler et al. 1962; Knoll et al. 2008) (Figure 4A), in keeping with a classic phytogeographic model (Vavilov 1951). Under this model, ancestrally chilling-sensitive African sorghums would have adapted to cold upon dispersion to temperate regions in central Asia and northern China (c. 800 years ago) due to derived alleles (Kimber 2000). However, our finding that chilling tolerance alleles coinherited with the ancestral wildtype alleles of classical tannin and dwarfing genes, which are widespread in both African and Chinese sorghums, suggests this model may be incorrect.

Instead, a revised model for derived chilling sensitivity of US sorghum and inadvertent selection may be more parsimonious (Figure 4B). Under this model, the African sorghums introduced into the US harbored basal chilling tolerance, but chilling sensitivity was inadvertently selected along with loss-of-function alleles at Tan1 and Tan2 (from African standing variation), and Dw1 and Dw3 (from de novo mutations in US) (Multani et al. 2003; Morris et al. 2013b; Hilley et al. 2016). Supporting this revised model, 38 RILs selected for agronomic suitability by the sorghum breeder (R.P.) were fixed for the chilling-susceptibility alleles (at qSbCT09.57 and qSbCT07.59) that are coinherited with desired Dw1 and Dw3 alleles, respectively (Table S8). Thus, coinheritance of chilling susceptibility with desired traits likely stymied >50 years of chilling tolerance breeding in this crop (Stickler et al. 1962; Tiryaki and Andrews 2001; Yu and Tuinstra 2001; Knoll and Ejeta 2008; Burow et al. 2010; Kapanigowda et al. 2013).

A genotype-to-phenotype modeling approach, which couples genetic and ecophysiologic modeling, can help assess the potential value of genotypes in a crop’s target population of environments (Cooper et al. 2014). Preliminary ecophysiologic modeling suggests that (were it not for chilling sensitivity) a standard grain sorghum hybrid could escape drought and have higher yields (~5%) if planted 30–60 days early (Figure S24). The improved power and resolution with the chilling NAM provides several new paths to obtain chilling tolerance while bypassing undesirable characteristics from Chinese sorghum. Several chilling tolerance alleles (at qSbCT05.04, qSbCT07.10, qSbCT01.13, and qSbCT01.57) are not coinherited with undesirable alleles for tannins and height (Figure 3) and can be used directly in marker-assisted introgression. Complementary dominance of Tan1 and non-functional tan2 (Wu et al. 2012) can be exploited to develop chilling-tolerant sorghums that retain the nontannin phenotype. If the standard model is correct (Figure 4A), rare recombinants identified with high-density markers will decouple chilling tolerance alleles from undesirable wildtype alleles of tannin and dwarving genes and bypass undesirable coinheritance. If the revised model is correct (Figure 4B), antagonistic pleiotropic effects could be bypassed with novel tannin biosynthesis mutations to disrupt tannin production in Tan1 Tan2 chilling-tolerant background and novel dwarfing mutants (Jiao et al. 2016) in Dw1 Dw3 chilling-tolerant background.

Conclusions

Genetic tradeoffs caused by linkage drag have long been appreciated by geneticists and breeders (Zhu et al. 2018; Cobb et al. 2018). More recently, genetic tradeoffs due to antagonistic pleiotropy or conditional neutrality (Anderson et al. 2011) have been revealed by positional cloning of key agronomic genes (i.e., those under strong selection in 20th century breeding programs). For instance, antagonistic pleiotropic
effects were identified for key improvement alleles of rice *semi-dwarf*1 (Li et al. 2018) and tomato *jointless* (Soyk et al. 2017). In elite rice germplasm, conditional neutrality led to unintentional fixation of a drought-susceptibility allele at *Deeprooting*1 (Uga et al. 2013). Similarly, our findings suggest that strong selection for nontrivial alleles (*tan1* and *tan2*) and dwarfing alleles (*dwl* and *dws*) in grain sorghum in the 20th century inadvertently resulted in the loss of early-season chilling tolerance, due either (i) to tight repulsion-phase linkage of desired alleles (Figure 4A) or (ii) antagonistic pleiotropic effects of desired alleles on chilling susceptibility (Figure 4B). Given increasing evidence of genetic tradeoffs for genes under strong directional selection, characterizing both the genetic architecture and molecular basis of adaptive variation will be critical to guide genomics-enabled breeding and understand adaptive mechanisms.

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