Genome-Wide Association Mapping of Grain Mold Resistance in the US Sorghum Association Panel

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ABSTRACT Sorghum [*Sorghum bicolor* (L.) Moench] production in warm and humid regions is limited by grain mold disease, which can be caused by a complex of >40 pathogenic and opportunistic fungi. The identification of resistant plants within temperate-adapted germplasm is imperative for the development of better-adapted varieties. The performance of 331 accessions from the previously genotyped sorghum association panel (SAP) was evaluated in four tropical environments. Only 18 accessions showed low seed deterioration and high emergence rates. The resistant accessions showed high variation in seed tannin contents and panicle shape, indicating that grain mold resistance is not associated with a single phenotypic trait. Seed mycoflora analysis recovered pathogenic fungi *Curvularia lunata*, *Fusarium thapsinum*, and *F. semitectum* in both resistant and susceptible accessions. By genome-wide association scans using 268,289 single nucleotide polymorphisms (SNPs), we identified two loci associated with low seed deterioration and another associated with emergence rate. Candidate genes within these loci included one *R* gene (*Sobic.08G132000*) and two genes (*Sobic.01G349300* and *Sobic.10G222400*) with domains associated with systemic acquired resistance, suggesting that resistance involved pathogen recognition and downstream signaling cascades. This study provides insight into the genetic control of grain mold resistance as well as valuable accessions for breeding programs in temperate environments.

Abbreviations: BTB/POZ, broad-complex, tramtrack, and bric-a-brac/pox virus and zinc finger; Chr., chromosome; ECMLM, enriched compressed mixed linear model; GBS, genotyping-by-sequencing; GWAS, Genome-wide association studies; LRR-NBS, leucine-rich repeat–nucleotide-binding site; MFS, major facilitator superfamily; NPGS, National Plant Germplasm System; QQ, quantile–quantile; SAP, sorghum association panel; SAR, system-acquired resistance; SKCS, single-kernel characterization system; SNP, single nucleotide polymorphism.

CORE IDEAS

- The sorghum association panel has very few grain mold-resistant accessions.
- Resistance genes were associated with pathogen recognition and the SAR response.
- Resistant accessions may be used to develop new grain mold-resistant varieties.

Sorghum is an important dietary staple cereal in the world together with maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and barley (*Hordeum vulgare* L.) (http://faostat.fao.org/site/339/default.aspx). In regions like Africa and India, the crop serves as vital cereal grain in their nutrition (Dahlberg, 2000). Today, food-grade sorghum is an important gluten-free alternative to wheat, and the identification of varieties with high concentrations of antioxidants has applications in the food industry (Awika and Rooney, 2004). Sorghum production regions include the warm and humid southeastern United States and Caribbean (USDA, 2014), where fungi diseases such as anthracnose and grain mold are among the most limited yield factors.

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Grain mold is a disease caused by multiple pathogenic and opportunistic fungi that began to infect and colonize flowers tissues prior to grain maturity (Bandyopadhyay et al., 2000). The most predominant fungi recovered in affected sorghum production regions worldwide are *Fusarium* spp., *Curvularia* spp., *Alternaria* spp., and *Colletotrichum* spp. (Das et al., 2012; Williams and Rao, 1981). The disease can be divided into two stages: (i) grain mold caused by the infection and colonization of spikelet tissues prior to grain maturity and (ii) weathering caused by fungi that colonize grain during caryopsis maturation and deterioration by environmental condition (Bandyopadhyay et al., 2000). This complex of mold and weathering occurring from anthesis to harvest is known as grain mold disease and reduces sorghum grain yield and quality. The large number of fungi involve in the disease constrained its control to the use of resistant cultivars (Thakur et al., 2007). The National Plant Germplasm System (NPGS) of the USDA has the largest worldwide sorghum collection, with >41,860 accessions from 114 countries, of which, ~80% is tropical exotic germplasm. The recent screening of this collection identified grain mold resistance sources among subsets from Sudan (Prom and Erpelding, 2009), Burkina Faso (Cuevas et al., 2016), Uganda (Prom et al., 2011), Senegal (Cuevas et al., 2018b), and western and central African countries (Ratnadass et al., 2003). The use of this material is limited by photoperiod sensitivity and a paucity of information on the genetic basis of resistance. Therefore, it is necessary to identify grain mold resistance in temperate-adapted germplasm and pinpoint the genomic regions involved in the resistance response to obtain immediately usable germplasm and genomic resources for breeding programs.

Sorghum is a genetically diverse crop classified into five major races (Kafir, Durra, Bicolor, Caudatum, and Guinea) that evolved from multiple independent redomestication process (Harlan and Dewet, 1972). Each sorghum race has particular traits in their inflorescence types and kernel morphology that provide advantage within specific environments (Snowden, 1936). The SAP (Casa et al., 2008) includes the five major races and was assembled as a germplasm resource to studying and discovering economic important traits in breeding programs. In this regard, the SAP was genetically characterized through genotyping-by-sequencing (GBS) (Elshire et al., 2011) to identify >250,000 SNPs (Morris et al., 2013a). Today, the SAP is an important germplasm source used for the study of multiple economic important traits. Genome-wide association studies (GWAS) using these resources have resulted in the discovery of multiple SNP–trait associations that provide insight into its genetic architecture (Adeyanju et al., 2015; Boyles et al., 2016; Chen et al., 2017; Cuevas et al., 2018a; Hayes et al., 2015; Morris et al., 2013b; Rhodes et al., 2014, 2017),

Because tropical regions provide optimal environmental conditions (i.e., high humidity and adequate temperatures) for the identification of fungal disease-resistant germplasm, we evaluated the grain mold resistance response of the entire SAP across four tropical environments to identify 18 resistant accessions. Genome-wide association scans were used to identify resistance-related loci. Our results are useful for breeding programs focused in the development of new resistant varieties as well as a suitable resistant reference set to compare and assess new resistance sources from the NPGS collection when this germplasm is evaluated in tropical environments.

MATERIALS AND METHODS

Germplasm and Field Experiment

Three hundred thirty-one accessions from the SAP (Casa et al., 2008) consisting of 212 temperate-adapted germplasm, 36 breeding lines, and 83 cultivars were evaluated for grain mold and weathering resistance to identify the most valuable germplasm for replicated testing (Supplemental Table S1). The SAP accessions and susceptible (BT×623 and RT×430) and moderately resistant (Sureño) controls were planted in research farms at the USDA–ARS Tropical Agriculture Research Station at Isabela and Mayaguez, PR, in 2014 (Experiment 1). At both locations, a completely randomized design with a single replication in each environment was used, with plots measuring 1.8 m in length with 0.9 m between rows.

Data from the two locations in Experiment 1 were used to select a subset of 42 accessions with either an average seed deterioration of ≤2.5 (on a 5-point scale) or an emergence rate of ≥85.0% for an additional replicated trial at Isabela, PR (Experiment 2). This subset, reference checks, and 10 susceptible accessions from the SAP were planted in 2015 in a randomized block design consisting of four blocks with plots of 3.0 m in length and 0.9 m between rows.

The data from Experiments 1 and 2 were used to select a subset of 22 accessions with an average seed deterioration of ≤2.5 or emergence rate of ≥85.0% for another replicated trial at Isabela, PR (Experiment 3). This subset, reference checks, and two susceptible accessions were planted in 2016 in a randomized block design consisting of eight blocks with plots of 3.0 m in length and 0.9 m between rows. The experiment was set up inside a frame structure with 30% shade cover and an overhead mist irrigation system to maintain the relative humidity at >85% most of the time. Temperature and relative humidity were recorded at intervals of 30 min throughout the experiments using an Onset HOBO U23 Pro v2 located within research plots (Supplemental Table S2).

Grain Mold Response

For Experiments 1 and 2, the most prevalent fungi in the region (*Curvularia lunata*, *Fusarium thapsinum*, and *F. semitectum*, according to Erpelding and Prom [2006]) were used for inoculations. The fungi were previously isolated from infected seeds, maintained on sterile filter papers at –20°C, and cultured on half-strength potato-dextrose agar at 25°C with a 12 h photoperiod for 7 to 14 d before the inoculations. A conidial mixture was prepared daily combining ~50 mL of each conidial suspension into

a 1.9 mL spray bottle filled with distilled water (Prom et al., 2011). The precise final conidial concentration of each fungus was not determined, nevertheless, the inoculations assurance the presence of fungal spores during anthesis and increase the humidity. Three to five panicles located at the start, middle, and end of the plot with uniform flowering times were inoculated daily from the first to the last anthesis day and covered with mesh bags for exposure to other environmental pathogens and to avoid damage by birds. In Experiment 3, three to five panicles were selected and protected as described in Experiment 2 without been inoculated. Panicles were hand harvested 35 to 40 d after anthesis, dried, and threshed using a single-plant thresher (Almaco Single Plant and Head Thresher; Allan Machine Company).

The grain mold resistance response was determined based on visual seed mold and weathering degradation (hereafter referred to as seed degradation) and emergence (Prom et al., 2011). Approximately 400 seeds from each panicle were assessed for seed degradation using a 5-point visual scale (Isakeit et al., 2008; Thakur et al., 2007), where 1 indicates seed is bright with no mold and no discoloration resulting from weathering; 2 indicates seed is not as bright and has little or no mold but has some discoloration (1–10% molded kernels); 3 indicates seed is not bright, with some mold and some discoloration (11–25% molded kernels); 4 indicates seed is almost entirely covered in mold, and the pericarp is degraded (26–50% molded kernels); and 5 indicates seed is covered entirely with mold, and the pericarp is degraded and looks dead (>50% molded kernels). The emergence rate was determined based on the number of seedlings observed after 10 d from 30 planted seeds from each panicle (i.e., 90 to 150 seeds from each experimental unit). The seeds were planted in flats containing Metro Mix 200 potting medium and incubated in a greenhouse at room temperature.

Compositional Analysis of Seeds

The composition of whole seeds harvested from Experiment 3 was analyzed by near-infrared spectroscopy (Dykes et al., 2014). Based on near-infrared calibration curves, the percentages of protein, fat, fiber, ash, total phenols (mg gallic acid equivalent g^{-1}), condensed tannin (mg catechin equivalents g^{-1}), and 3-DOA (absorbance mL^{-1} g^{-1}) were estimated for each accession. The grain hardness index was estimated using a single-kernel characterization system (SKCS), where 100 seeds per plot were scanned using a SKCS-4100 (Perten Instruments) controlled by MicroSoft Windows SK4100 v. 2.1.0.1 (Bean et al., 2006).

Mycoflora Analysis

An internal mycological survey was performed using the seeds from Experiment 3 as described by Prom (2004). Twenty seeds from seven sorghum lines (two resistant [Red Amber and SC15], three moderately susceptible [Della, Pink Kaffir, and El Mota], and two highly susceptible [BT×623 and RT×430]) were randomly selected for mycological survey (i.e., a total of 140 seeds). Seven fungal groups were initially identified based on morphological characters. Later, three samples from each fungal group were analyzed by a sequencing analysis of the β-tubulin 2 gene to determine the fungal species. Fungal DNA was isolated using the ZR Fungal–Bacterial DNA MicroPrep Kit (Zymo Research), and β-tubulin 2 was partially amplified and sequenced by BigDye terminator chemistry (SeqWright Genomic Services; A NeoGenomics Company) with previously designed primers (Glass and Donaldson, 1995). Sequence chromatograms were examined using SEQUENCHER (version 4.1; Gene Codes Corporation) and evaluated with other homologous sequences using the NCBI Basic Alignment Search Tool and Fusarium ID database (http://isolate.fusariumdb.org/blast.php).

Phenotype Analysis

A mixed linear model with locations treated as fixed effects and accession as a random effect was used to estimate the repeatability of seed degradation and emergence rate for Experiment 1 with the following formula:

$$R^2 = s^2_G/[s^2_G + (s^2_E/L)],$$

where G represents the accession, L is the location, and E is an error term (Bernardo, 2002). The analyses of variance for Experiments 2 and 3 were based on a mixed linear model with accessions treated as fixed effects and block as a random effect. The performance of the accessions and populations was estimated based on least square means and compared using the Tukey–Kramer honest significant difference test.

Genotype-by-Sequencing

Genotype information used in this study is a community resource generated for the entire SAP (Morris et al., 2013a) and was improved by the alignment of raw GBS data to the most recent version of the sorghum reference genome (version 3.1; www.phytozome.net, accessed 15 Feb. 2018) (Boyles et al., 2016). Missing genotype data were imputed using fastPhase (Scheet and Stephens, 2006) with 10 independent starts of the expectation-maximization algorithm. The imputed genotype data set was filtered for the minor allele frequency (>0.05) and missing data (<0.20) to retain 268,289 SNPs for the association analysis.

Population Structure

The SAP population structure has been described in previous publications (Casa et al., 2008; Cuevas et al., 2018a).

Phylogenetic Analysis

Pairwise genetic distances among grain mold-resistant accessions were calculated based on identity by state as implemented in TASSEL 5.0 (Glaubitz et al., 2014) using 11,609 previously identified unlinked SNPs (Cuevas et al., 2018a). Clustering analysis was based on neighbor-joining method and phylogenetic tree visualized using Interactive Tree of Life (Letunic and Bork, 2011).

Genome-Wide Association Analysis

The enriched compressed mixed linear model (ECMLM) (Li et al., 2014) was implemented to analyze quantitative data (i.e., seed degradation and emergence) via the GAPIT package in R (Lipka et al., 2012). The first three principal components were included as covariates and the kinship matrix was calculated as described by VanRaden (2008) to control for population structure and family relatedness. Based on the visual inspection of log quantile–quantile (QQ) *p*-value plots, all analyses had adequate controls for both population structure and familial relatedness, indicating the effective control of false positive errors (Supplemental Fig. S1). Manhattan and QQ plots were visualized using the R package qqman (Turner, 2014). The empirical significance thresholds for the association analyses of seed degradation [$-\log_{10}$ (*p*-value) = 5.70] and emergence rate [$-\log_{10}$ (*p*-value) = 5.49] were calculated with 1000 permutations for an experiment-wise error of $p = 0.05$.

Targeted Resequencing

Candidate genes associated with SNPs identified by GWAS were determined using the most recent version of the sorghum reference genome (version 3.1; www.phytozome.net, accessed 15 Feb. 2018). Functional annotations and tissues with high expression levels of each candidate gene were retrieved from the Gene Atlas Tissues Sample available in Phytozome (www.phytozome.net, accessed 15 Feb. 2018). The candidate genes identified by GWAS were partially sequenced using a subset of accessions (resistant and susceptible) via BigDye terminator chemistry (SeqWright Genomic Services; A NeoGenomics Company) to verify and identify additional valuable SNPs for marker development. The primers were designed using Primer3 (Supplemental Table S3) (Koressaar and Remm, 2007), and the sequence chromatograms were examined using SEQUENCHER (version 4.1; Gene Codes Corporation).

RESULTS

Grain Mold Resistance

We found that the majority of accessions in the SAP are highly susceptible to grain mold, with means of 3.9 and 60.7% for the seed degradation score and emergence rate, respectively (Experiment 1; Supplemental Table S1). A total of 26 accessions showed seed degradation scores of ≤2.5, and 45 accessions had an emergence rate of ≥85.0%; 20 exhibited both low seed degradation and a high emergence rate. The estimated repeatabilities were 0.69 and 0.60 for seed degradation and emergence rate, respectively, indicating that the majority of the total phenotypic variation is due to genotypic variation.

The subset of 42 accessions that were evaluated for a second year (Experiment 2) averaged 2.3 and 64.6% for the seed degradation score and emergence rate, respectively (Table 1). A total of 27 accessions exhibited seed degradation scores of ≤2.5, seven accessions had an emergence rate of ≥85.0%, and six exhibited both low seed degradation and a high emergence rate. When Experiments 1 and 2 were combined, 22 accessions had an average seed degradation score of ≤2.5 and emergence rate of ≥85.0%. These 22 accessions were subjected to environmental conditions that were most favorable for the development of grain mold disease.

The subset of 22 accessions that were evaluated for a third year (Experiment 3) averaged 2.7 and 63.9% for the seed degradation score and emergence rate, respectively (Table 1). Nine accessions demonstrated seed degradation scores of ≤2.5, and one had an emergence rate of ≥85.0%. We found that accessions with seed degradation scores of ≤2.1 and emergence rates of ≥71.2% were the most grain mold resistant. Five accessions (SC605, SC782, Lian Tang, Acme broom, and SC623) exhibited low seed degradation, four (SC1494, SC598, Della, and SC224) had high emergence rates, and nine (Red Amber, SC15, 6550 Sumac, SC13, Keller, Kansas Orange, Rox Orange, SC309, and SC609) exhibited low seed degradation and high emergence rates (i.e., totally resistant). Because there was limited overlap in the accessions that exhibited both low seed degradation and high emergency rates, these two aspects of resistance (grain mold and weathering) are almost certainly controlled by different physiological mechanisms; these mechanisms could be complementary and might be combined into a single variety to enhance grain mold resistance. Therefore, the 18 accessions with the lowest seed degradation scores and highest emergence rates could be used in breeding programs to develop new grain mold resistance cultivars.

Originally, the population structure of the SAP was classified into 10 populations associated with sorghum races and geographic locations (Casa et al., 2008). One of these populations, the Sudanense–Broomcorn–Guinea group, had significantly lower seed degradation ($X = 2.9$) than any other subgroup (Table 2). A second population, the Guinea–Caudatum–Bicolor (E. Africa–India) group, had the highest emergence rate in the SAP (mean = 72.3%) (Table 2). When individuals were grouped by race only (without regard to geographic subpopulations), we did not detect a relationship between population structure and resistance. This strongly suggests that geographic origin and the exposure of plants in certain locations to pathogens has historically driven mold resistance. This result is particularly important since panicle architecture (i.e., race) has been cited as an important adaptation in humid environments, but we found that resistant varieties can occur in any race, likely because so many different characteristics can influence grain mold tolerance.

Understanding the genetic diversity among grain mold-resistant cultivars could be useful for the development of new resistant varieties. The genetic distance and unrooted neighbor-joining tree (Fig. 1) found resistance accessions were representative of the genetic diversity present in SAP. In fact, resistance accessions belonged to five populations when the SAP was classified into 10 populations, with Kafir as the highly most represented population, with seven accessions. By contrast, when the SAP was grouped by race, only 10 accessions were classified as admixed: three Caudatum (SC309, Liang Tang Ai, and SC605), three Kaffir (SC782,

Table 1. Grain mold resistance response of 23 accessions from the US sorghum association panel (SAP).

Name	Tannin†	Kernel appearance‡	Isabela (2014)		Mayaguez (2014)		Isabela (2015)		Isabela (2016)		Overall	
			Seed§	Emer.¶	Seed	Emer.	Seed	Emer.	Seed	Emer.	Seed#	Emer.#
Red Amber	49.2	Brown	2.5	96.7	1.5	100.0	1.0	91.0	2.0	82.1	1.4a	88.2a
SC15	11.4	Brown	1.5	91.7	2.0	93.3	2.0	84.4	2.4	88.1	2.1a	86.5a
Sumac	42.8	Brown	1.5	90.0	2.0	93.3	1.8	87.0	2.4	78.8	2.0a	85.0a
SC13	09.4	Brown	2.0	96.7	1.5	93.3	1.5	83.0	2.7	84.2	1.9a	84.1a
Keller	26.9	Brown	2.5	96.7	2.0	80.0	1.6	84.7	2.7	75.0	1.9a	82.1a
Kansas Orange	23.0	Brown	2.0	98.3	2.0	71.7	1.5	93.8	2.4	67.5	2.0a	81.5a
Rox Orange	37.9	Brown	2.0	96.7	2.0	96.7	1.6	83.6	2.9	66.3	2.1a	78.6a
SC309	00.0	Brown	4.5	83.3	2.5	98.3	1.4	77.7	3.0	77.5	2.0a	78.6a
SC609	17.1	Brown	3.0	96.7	1.5	93.3	1.3	80.5	2.5	59.4	1.7a	75.3a
SC605	37.4	Brown	2.0	60.7	1.0	73.3	1.2	57.0	1.9	33.1	1.5a	49.0
SC782	43.1	Brown	3.0	86.7	1.0	76.7	1.2	66.8	2.8	60.6	1.8a	66.1
Lian Tang Ai	30.0	Brown	1.5	90.0	2.5	60.0	1.8	48.1	1.4	51.7	1.8a	48.3
Acme Broomcorn	61.4	Brown	1.5	96.7	1.0	90.0	2.1	58.8	2.2	45.9	2.0a	57.6
SC623	07.0	Brown	2.0	93.3	2.0	58.3	2.1	68.0	2.1	63.1	2.1a	66.4
SC1494	09.5	White	4.0	91.7	4.0	95.0	2.0	91.8	3.5	59.2	2.7	81.5a
SC598	16.2	Brown	3.0	100.0	2.5	93.3	2.6	85.4	3.1	66.0	2.8	80.1a
Della	25.0	Brown	4.0	88.3	3.5	93.3	2.0	74.5	2.7	82.4	2.5	79.4a
SC224	36.2	Brown	3.0	96.7	1.5	98.3	2.1	69.1	2.7	68.1	2.3	71.2a
Sureño	00.0	White	1.5	91.7	5.0	15.0	2.0	77.3	2.9	46.4	2.4	65.7
SC295	01.3	White	3.0	98.3	2.5	76.7	1.1	67.0	3.2	55.6	3.1	63.5
SC614	03.9	White	3.0	86.7	3.0	90.0	1.9	57.7	3.4	56.4	2.4	60.0
Marupantanse	02.7	White	3.5	88.3	3.5	91.7	2.2	53.9	3.6	52.1	2.7	56.6
MR732	01.3	White	3.5	93.3	3.5	85.0	1.8	89.6	3.3	34.6	3.0	54.3
Pink Kafir	01.2	White	4.5	91.7	3.5	95.0	1.9	50.2	3.6	33.8	2.6	48.0
Rio	14.0	White	4.5	96.7	5.0	80.0	2.4	85.3	3.8	69.6	3.5j	76.7
El Mota	11.2	White	3.5	80.0	3.5	91.7	NA††	NA	4.1	41.3	4.0j	17.5i
BT×623 (Control)	09.4	White	4.0	50.0	5.0	33.3	3.0	28.0	3.7	6.1	3.2	22.1i
RT×430 (Control)	00.0	Yellow	5.0	33.3	5.0	23.8	4.1	2.3	3.0	0.0	4.1j	05.5i

† Condensed tannins based on milligrams of catechin equivalents (CE) g^{-1}; values <10 generally indicate low or nontannin genotypes.

‡ Visual color of kernels resulting from the interactions of pericarp color, mesocarp thickness, pigmented testa, and spreader treats.

§ Seed mold and weathering degradation based on a 1-to-5 scale, where 1 indicates no mold and 5 is >50% molded kernels and pericarp is degraded.

¶ Emergence rate based on the percentage of seedlings observed after 10 d in flats containing Metro Mix 200 potting medium.

Within a column, means with the same letter are not significantly different based on Tukey-Kramer tests.

†† NA, not applicable.

Table 2. Grain mold resistance of 335 accessions from the US sorghum association panel (SAP).

Population structure based on 47 simple sequence repeats (Casa et al., 2008)				Population structure based on 1000 single nucleotide polymorphisms			
		Seed				Seed	
Subpopulations	n	Seed ± SD†	Emer. ± SD‡	Subpopulations	n	Seed ± SD†	Emer. ± SD‡
Sudanense–Broomcorn–Guinea	14	2.9 ± 0.2a	66.2 ± 5.4ab	Caudatum	75	3.9 ± 0.1a	61.5 ± 2.4a
Guinea–Caudatum–Bicolor (E. Africa–India)	23	3.5 ± 0.2ab	72.3 ± 4.2a	Guinea	20	4.1 ± 0.2a	51.4 ± 4.7a
Caudatum–Bicolor	19	3.7 ± 0.2bc	68.1 ± 4.6ab	Durra	31	4.3 ± 0.2a	55.6 ± 3.8a
Kafir	63	3.8 ± 0.1bc	67.5 ± 2.5ab	Kafir	47	4.1 ± 0.1a	60.7 ± 3.1a
Zerazera–Caudatum	46	3.9 ± 0.1bc	59.7 ± 3.0abc	Admixed	157	3.8 ± 0.1a	62.7 ± 1.7a
Caudatum	38	4.0 ± 0.1bc	58.9 ± 3.3abc	–	–	–	–
Caudatum–Kafir	33	4.1 ± 0.1bc	63.9 ± 3.5ab	–	–	–	–
Durra	44	4.2 ± 0.1bc	52.7 ± 3.0bc	–	–	–	–
Guinea–Caudatum (W. Africa)	24	4.2 ± 0.2c	53.7 ± 4.1bc	–	–	–	–
Milo–Feterita	27	4.3 ± 0.2c	46.1 ± 3.9c	–	–	–	–

† Seed grain mold degradation based on a 5-point scale, where 1 indicates no mold and 5 indicates >50% molded kernels and pericarp is degraded. Means with the same letter are not significantly different based on Tukey-Kramer tests.

‡ Emergence refers to the percentage of seedlings observed after 10 d in flats containing Metro Mix 200 potting medium. Within a column, means with the same letter are not significantly different based on Tukey-Kramer tests.

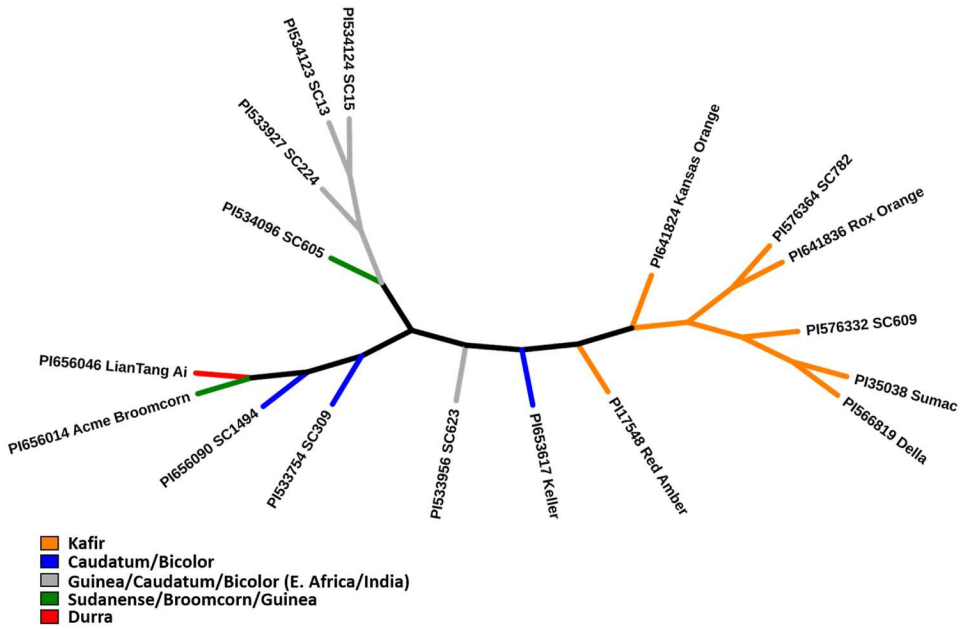

Fig. 1. Unrooted neighbor-joining tree of 17 grain mold resistance accessions present in the sorghum association panel. Each branch includes the plant identification number and cultivar name, and the colors represent the populations according to Casa et al. (2008).

SC609, and Della), and one Durra (Acme Broomcorn). The genetic diversity indicates that resistant accessions resulted from the combination of multiple sources of resistance in different sorghum races and geographic locations.

Additional factors that influence grain mold resistance include condensed tannins and certain phenolic compounds such as flavan-4-ols (Waniska, 2000). While recent studies showed that tannins may be beneficial for human health because of their high antioxidant capacity, high levels of these compounds also reduce animal feed intake and feed conversion efficiencies (Dixon et al., 2005). Based on the whole-seed compositional analysis and grain hardness index for 26 accessions, most of these traits did not differ between resistant and susceptible cultivars (Table 3). However, the resistant accessions exhibited higher average levels of tannins (27.2 mg catechin equivalents g^{-1}), total phenols (16.2 mg gallic acid equivalent g^{-1}), and 3-DOA (72.5 absorbance mL^{-1} g^{-1}) than did the susceptible accessions (12.3, 9.2, and 37.5, respectively) (Table 1, 3). Based on tannin contents, resistance accessions were grouped into 8 to 10 categories with contents ranging from 0.0 (SC309) to 61.4 (Acme broom). Likewise, based on total phenols, resistant accessions were grouped into nine categories with values ranging from 5.2 (SC309) to 27.8 (Acme broom). The high variation, even among resistant accessions, strongly suggests that while these compounds have an important function in the resistance of some lines, other mechanisms must also control the resistance response. Thus, the variation among resistant accessions identified here offers promise for breeding programs, but their use will be limited to specific markets.

Multiple fungi are associated with sorghum grain mold disease (Thakur et al., 2007). In Puerto Rico, the most prevalent fungi are *Curvularia lunata* and *Fusarium* spp. (Erpelding and Prom, 2006). In a mycological survey of seven accessions, *Curvularia lunata*, *Fusarium semitectum*, and *F. thapsinum* were the most frequently recovered fungi (Supplemental Table S3). Nevertheless, seed mycoflora varied among resistant (Red Amber and SC15) and susceptible (BT×623 and RT×430) accessions, and multiple fungi were most often recovered in the susceptible accessions. The presence of pathogenic fungi in resistant accessions suggests that infection occurs, but the resistance mechanism limits its growth or impact on the developing seed.

Genome-Wide Association Analysis

In a genome-wide association scan for seed degradation, we detected two loci on chromosomes (Chr.) 1 [Chr.1: 63,891,513; –log (*p*-value) = 6.18] and 8 [Chr.8: 55,843,085; –log (*p*-value) = 6.88] (Fig. 2). The locus on chromosome 1 was located ~5 Mb upstream of the sorghum *y1* (*yellow seed1*) locus associated with seed pericarp pigmentation (Boddu et al., 2005). The estimated allelic effects of the loci were −0.39 and −0.31, respectively, based on the 5-point seed score. In a genome-wide association scan for emergence rate, we detected one locus on chromosome 10 [Chr.10: 56,467,067; –log (*p*-value) = 5.86] (Fig. 3). The estimated allelic effect was a 9.53% increase in the seed emergence rate. These loci explain only a small portion of the total observed variation, indicating that other phenotypic mechanisms and related resistance loci are present. Loci that we did not detect in this study could be related to other grain mold-resistant components.

Candidate Genes in Grain Mold Resistance Loci

We identified candidate resistance genes in the three grain mold resistance loci (Table 4). The locus on chromosome 1 consisted of one SNP located within the coding region of the putative gene *Sobic.001G349300* (major facilitator

Table 3. Grain quality of 26 accessions from the US sorghum association panel (SAP) evaluated for grain mold resistance under high humidity at Isabela, Puerto Rico.

ID	Name†	Ash	Fat	Fiber	DOA‡	Starch	Protein	Phenols§	Hardness¶
		%			absorbance mL^{-1} g^{-1}	%		mg (GAE) g^{-1}	
PI 17548	**Red Amber**	1.1	2.3	1.7	78.1	67.4	12.0	24.1	76.2
PI 534124	**SC15**	1.1	1.8	1.9	71.0	66.2	12.6	12.2	77.5
PI 35038	**Sumac**	1.0	2.1	1.8	83.7	68.3	12.3	22.0	71.8
PI 534123	**SC13**	1.1	1.9	2.1	68.1	65.8	11.8	10.6	70.7
PI 653617	**Keller**	1.1	2.4	1.8	52.1	68.3	09.8	14.4	87.4
PI 641824	**Kansas Orange**	1.1	2.1	1.8	60.8	67.0	10.7	14.0	63.2
PI 641836	**Rox Orange**	1.0	2.2	1.7	78.2	67.2	11.7	19.0	77.8
PI 533754	**SC309**	1.2	1.5	1.8	53.7	66.9	09.9	05.2	74.1
PI 576332	**SC609**	1.2	2.1	1.6	42.9	67.5	10.9	11.0	75.9
PI 534096	*SC605*	1.2	2.6	1.9	94.1	65.2	13.8	21.4	66.0
PI 576364	*SC782*	1.0	2.1	1.5	79.3	66.9	13.0	21.0	101.9
PI 656046	*Lian Tang Ai*	1.2	2.1	1.5	53.1	66.9	10.7	16.1	82.8
PI 656014	*Acme Broomcorn*	1.2	2.5	1.7	97.4	64.0	15.4	27.8	78.0
PI 533956	*SC623*	1.0	2.2	2.1	112.0	66.7	10.9	12.2	72.3
PI 656090	***SC1494***	1.0	1.3	1.8	36.8	71.3	07.7	08.9	15.6
PI 576337	***SC598***	1.1	1.8	1.8	78.1	66.4	11.0	12.9	77.1
PI 566819	***Della***	1.1	2.7	1.8	62.1	67.7	12.2	15.3	84.1
PI 533927	***SC224***	1.1	2.7	1.8	38.4	64.5	13.4	18.1	95.1
PI 561472	Sureño	1.3	2.1	1.9	32.6	67.1	10.0	03.9	91.5
PI 656093	SC295	1.2	1.2	1.5	23.4	70.1	08.0	04.8	91.5
PI 533940	SC614	1.2	1.4	1.4	28.8	68.8	08.2	04.2	83.7
PI 656049	Marupantanse	1.1	1.0	1.6	33.0	70.8	07.0	04.2	86.3
PI 656051	MR732	1.2	2.1	1.7	28.9	68.3	09.1	03.7	84.5
PI 655972	Pink Kafir	1.1	1.1	1.8	17.4	70.7	07.6	03.8	82.2
PI 651496	Rio	1.1	0.5	1.7	11.4	71.5	06.7	08.3	65.0
PI 656035	El Mota	1.1	0.3	1.6	23.5	71.3	07.2	08.3	79.1

† Grain mold-resistant: bold text indicates low seed deterioration and high emergence rate; italic text (no bold) indicates low seed deterioration; italic and bold text indicates high emergence rate.

‡ DOA, 3-deoxyanthocyanidin content.

§ GAE, gallic acid equivalent.

¶ Grain hardness index based on the single-kernel characterization system.

superfamily [MFS]). Sixteen SNPs in linkage disequilibrium ($R^2 > 0.91$) were found in the locus on chromosome 8. These SNPs are distributed across the putative gene *Sobic.008G132000* (leucine-rich repeat–nucleotide-binding site [LRR-NBS]), including five in the 5′ untranslated region, one in the 3′ untranslated region, and 10 in the coding region. The other locus on chromosome 10 contained two SNPs in the coding region of the putative gene *Sobic.010G222400* (broad-complex, tramtrack, and bric-a-brac/pox virus and zinc finger [BTB/POZ] domain). Genes with similar functions are associated with plant immunity. Major facilitator superfamily is a new class of plant defense-related proteins that could be involved in (i) the export of antimicrobial compounds produced by plant pathogens, (ii) the export of plant-generated antimicrobial compounds, and (iii) potassium export and reuptake, as can occur in plant defense reactions (Simmons et al., 2003). Most resistant (*R*) genes contain LRR-NBS domains, which act as receptors of pathogen effectors to activate the signaling cascade for defense (Jones and Dangl, 2006). The BTB/POZ domain is an evolutionarily conserved and widely distributed motif in genes within the system-acquired resistance (SAR) response pathway (Wu et al., 2012b). The SAR response is an innate immune response that provides protection against a broad range of biotrophic pathogens by the induction of defense compounds such as salicylic acid. These candidate genes should be evaluated further to determine their exact functions in the grain mold resistance response.

Targeted Resequencing

A partial resequencing analysis of candidate genes in a subset of 31 accessions (17 resistant and 14 susceptible; Supplemental Table S3) validated SNPs from the GBS analysis and indicated the presence of multiple haplotypes. For the locus on chromosome 1 (*Sobic.001G349300*), we resequenced a 647 bp coding segment to detect six SNPs (validating three SNPs from GBS, Chr.1: 63,891,331, Chr.1: 63,891,419, and Chr.1: 63,891,513) and one insertion–deletion of 15 bp (Chr.1: 63,891,107–63,891,121). A total of six protein variants were identified based on six haplotypes. For the locus on chromosome 8 (*Sobic.008G132000*), we resequenced 610 and 700 bp segments in exon 1 and 2, respectively, to identify 11 SNPs (validating two SNPs from

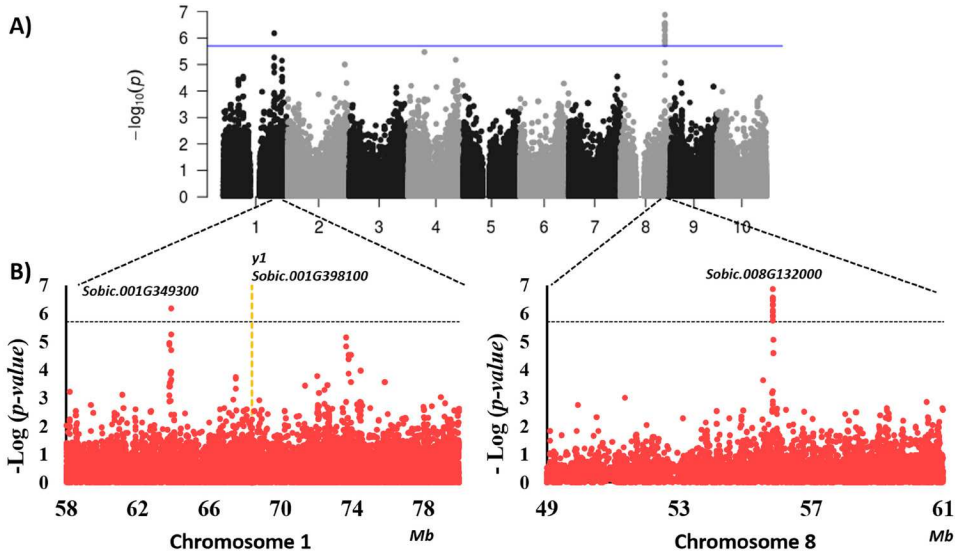

Fig. 2. Genome-wide association analysis of grain mold resistance in the sorghum association panel based on seed degradation. (A) Manhattan plots for enriched compressed mixed linear model and the significant threshold ($p < 0.05$) based on 1000 permutations. (B) Grain mold-resistant loci on chromosomes 1 and 8, where the dashed yellow line represents the location of the *yellow seed1* (*y1*) gene.

GBS, Chr.8: 55,840,374 and Chr.8: 55,841,513). We identified four haplotypes corresponding to four protein variants. For the locus on chromosome 10 (*Sobic.010G222400*), we resequenced a 560 bp segment that included the intron, exon 2, and the 3′ untranslated region to identify eight SNPs. Two protein variants were identified based on two haplotypes. The protein variants from these three genes were present in both resistant and susceptible accessions, indicating that the resistance response must involve interactions with other genes.

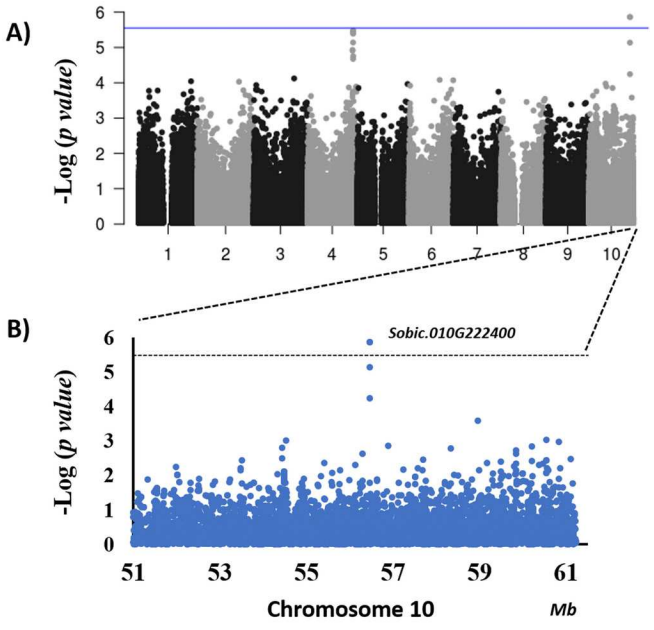

Fig. 3. Genome-wide association analysis of grain mold resistance in the sorghum association panel based on emergence rate. (A) Manhattan plots for enriched compressed mixed linear model and the significant threshold ($p < 0.05$) based on 1000 permutations. (B) Grain mold-resistant locus on chromosome 10.

DISCUSSION

The identification of grain mold resistance in temperate-adapted germplasm provides an opportunity to the development of new varieties. Two resistance sources used in breeding programs (Sureño and SC719-11E) are only moderately resistant or susceptible in high humidity environments (Cuevas et al., 2016; Erpelding, 2009; Prom et al., 2011). In the present study, the seed deterioration score and emergence rate of Sureño were 2.4 and 65.8%, respectively, indicating that it is moderately resistant and, in some cases, still susceptible under high humidity (Table 1). Therefore, the resistant accessions identified here are potential sources for sorghum breeders to develop new grain mold-resistant varieties. In this regard, the lines SC623 and SC1494 were also classified as resistant based on 2 yr of evaluation at Manhattan, KS, (Tomar, 2016). The limited resistant germplasm in the SAP and the high genetic relatedness among some accessions (e.g., Lian Tang Ai, Acme Broomcorn, and SC1494 [identity by state < 0.11]) also make it imperative to explore exotic sorghum germplasm for novel sources of resistance. Although numerous resistance sources have been identified by the screening of multiple subsets from the NPGS germplasm collection (Cuevas et al., 2016, 2018b; Erpelding, 2009; Prom et al., 2011), their introgression into temperate-adapted germplasm is required for sorghum breeding.

Sorghum races reflect local adaptation to particular environments and cultural practices (Smith and Frederiksen, 2000; Snowden, 1936). For instance, the open panicles of the Guinea race, which is originally from the humid western Africa, are often associated with increased resistance to grain mold and insect pressure. An assessment of the NPGS Senegalese germplasm composed of Guinea sorghum types revealed a high frequency of grain mold resistance (Cuevas et al., 2018b).

Table 4. Summary of grain mold resistance loci detected by the genome-wide analyses of seed deterioration and emergence rate using the sorghum association panel (SAP).

SNP genomic position	Allele	Allele frequency	Allele effect	Gene†	Annotated function†	High gene expression‡
			Seed deterioration			
Chr.1: 63,891,513	A	0.89	–	Sobic.001G349300	Major facilitator superfamily domain	Leaf sheath growing anthesis; leaf sheath growing floral initiation; panicle floral initiation; root bottom juvenile; seed imbibed grain maturity; stem mid internode grain maturity.
	G	0.11	–0.39			
Chr.8: 55,843,085	A	0.74	–	Sobic.008G132000	Leucine-rich repeat-containing protein; NB-ARC domain	Internode growing floral initiation; leaf sheath growing grain maturity; panicle upper anthesis; panicle floral initiation; peduncle floral initiation; leaf flag one internode grain maturity.
	G	0.26	–0.31			
			Emergence rate			
Chr.10: 56,467,067	A	0.85	–	Sobic.010G222400	BTB/POZ domain	Internode growing upper floral initiation; panicle floral initiation; peduncle floral initiation.
	G	0.15	9.53			

† Gene candidate and annotated function based on the sorghum genome version 3.1.

‡ High gene expression according to Phytozome v.12.1.

Six of the resistant accessions in the SAP belong to two populations with a Guinea genetic background, and their panicle shapes reflect the more open structure typical of this race. Nevertheless, we observed grain mold resistance in genotypes with a compact panicle shape in eight resistant accessions. Thus, grain mold resistance is a highly complex trait influenced by numerous panicle and kernel traits as well as multiple fungal defense genes.

High levels of condensed tannins in mature sorghum kernels are correlated with resistance to grain mold (Harris and Burns, 1973; Menkir et al., 1996). The biosynthesis of tannins is controlled by the *Tannin-1* gene on chromosome 4 (*Sb004G280800*); the nonfunctional alleles *tan1-a* and *tan1-b* lead to a lack of tannin accumulation (Wu et al., 2012a). Nevertheless, our GWAS did not indicate an association between *Tannin-1* and grain mold resistance. To investigate the effect of tannins on grain mold resistance, we used a GBS SNP (S4_62316425) in perfect linkage disequilibrium with the *G* deletion that is causative for the *tan1-a* allele (Morris et al., 2013b). We found that the functional allele *Tannin-1* is present at a high frequency in the SAP (0.79) despite the high frequency of susceptible accessions. However, we observed that accessions with the nonfunctional *tan1-a* allele were significantly more susceptible to grain mold (4.4 and 44.4% for seed deterioration and emergence rate, respectively) than the accessions with the functional allele *Tannin-1* (3.8 and 65.1% for seed deterioration and emergence rate, respectively). Previous results showed that, among Caudatum, Guinea, and Kafir races, numerous accessions have the *Tannin-1* allele but exhibit the no-tannin phenotype (Morris et al., 2013b). In fact, the concentration of condensed tannins among the 18 resistant accessions was variable (0.0 to 49.2 catechin equivalents g^{-1}; Table 1), even though they have the *Tannin-1* allele. Certainly, *Tannin-1* explains a portion of the variation in grain mold resistance, but its modest correlation with population structure in the SAP (Morris et al., 2013b) is overcorrected by the ECMLM GWAS analysis. Thus, grain mold resistance is determined by a combination of multiple factors, only one of which is the concentration of tannins.

Understanding the molecular mechanism underlying grain mold resistance could provide a basis for the development of high-throughput screening systems. Previous quantitative trait loci studies enable the identification of larger genomics regions that requires further fine mapping approaches to pinpoint candidate genes (Klein et al., 2001). The three loci identified in this study provide the first insight into the genetic basis of grain mold resistance in sorghum. Marker-assisted selection based on these three loci together with phenotype selection could increase the genetic gain in breeding programs if resistance accessions identified herein are used as parental germplasm.

Although multiple pathogens concurrently infect the grain, the most significant SNPs were located within an *R* gene (*Sobic.008G132000*) involved in pathogen recognition. This immune receptor gene may recognize a common effector molecule produced by different pathogens or its activation by a pathogen effector may lead to a robust disease resistance response. In tomato (*Solanum lycopersicum* L.), the immune receptor gene *Ve1* mediates resistance against multiple pathogens (de Jonge et al., 2012). Broad-spectrum resistance can be also achieved by the activation of defensive genes acting downstream of pathogen recognition (McDowell and Woffenden, 2003). Using the NCBI Conserved Domains database, we found that *Sobic.010G222400* has BTB/POZ and MATH (meprin and TRAF-C) domains, both of which are associated with plant disease resistance. The non-expressor of pathogenesis-related gene is a key regulator of SAR; it contains an N-terminal BTB/POZ domain, an ankyrin repeat domain, a C-terminal transactivation domain, and a nuclear localization sequence (Kuai et al., 2015). Both the ankyrin repeats and the BTB/POZ domain interact with a transcriptional activator to produce the defense response (Boyle et al., 2009; Zhang et al., 1999). The MATH domain is associated with resistance to potyviruses in *Arabidopsis thaliana* (L.) Heynh. (Cosson et al., 2010) and apricot (*Prunus armeniaca* L.) (Mariette et al., 2016). In *A. thaliana*, a MATH-TRAF protein controls the long-distance movement of three potyviruses. In apricot, a BTB/POZ–MATH–TRAF protein is associated with the *PPV1a* locus, conferring resistance to potyvirus *Plum Pox Virus*. Thus, it is possible that *Sobic.010G222400* is

involved in a type of hypersensitive reaction that restricts the growth and spread of pathogens, leading to resistance. The MFS domain in *Sobic.001G349300* can have broad functional specificities (Van Bambeke et al., 2000); however, its role in plant defense is very limited. In maize, the *Zm-Mfs1* gene is highly expressed postinfection by *Cochliobolus heterostrophus*, the causal agent of southern corn leaf blight (Simmons et al., 2003). This protein may function to export toxins produced by plant pathogens, although its role does not appear to be decisive for resistance. Further functional studies of these candidate genes are needed; however, our results strongly suggest that both SAR and *R* genes are involved in grain mold resistance.

CONCLUSION

The environmental conditions in this study were optimal to identify the most highly grain-mold-resistant accessions in the SAP. The phenotypic and genotypic variation among resistant accessions was not associated with a single sorghum race or subpopulation, indicating that grain mold resistance is influenced by a combination of resistance-related genes that evolved under different environmental conditions. We identified three novel candidate loci on chromosomes 1, 8, and 10 involved in the recognition of pathotype-associated molecular patterns and the SAR response, although functional genomic studies are required to establish their function during the resistant response. Within the SAP, there are lines with grain mold resistance that can be used in breeding programs. However, the limited number of resistant accessions and their overall phenotype may constrain the development of new varieties. At a minimum, the resistant cultivars can be used as reference germplasm in further evaluations of the NPGS sorghum collection to identify new sources of resistance.

Supplemental Information Available

Supplemental information is available with the online version of this manuscript.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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