Natural Variation and Genome-Wide Association Study of Antioxidants in a Diverse Sorghum Collection

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ABSTRACT

Consumption of polyphenol-rich food is associated with decreased risk of oxidative stress-related chronic diseases. Sorghum, a major food and feed cereal crop, has many polyphenol-containing accessions with high antioxidant activity in the grain. However, many of these polyphenol-containing accessions are not high-yielding or food-grade varieties. The natural variation in sorghum grain polyphenols can be used to develop high-yielding, health-promoting specialty food types through marker-assisted breeding. To identify new antioxidant-rich germplasm, we quantified antioxidant activity, total polyphenols, and condensed tannins in whole kernel flour in 266 accessions from the genetically and phenotypically diverse Sorghum Association Panel. Antioxidant activity, total polyphenols, and condensed tannins were in the ranges of 9.6–325.1 μmol of trolox equivalents (TE)/g, 0.8–18.8 g of gallic acid equivalents/kg, and 0–65.5 g of catechin equivalents/kg, respectively. Twenty-three accessions were rich sources of antioxidant activity, with PI534144 (SC84; 325.1 μmol of TE/g) and PI534117 (SC991; 237.0 μmol of TE/g) possessing the highest values. To identify quantitative trait loci associated with sorghum grain antioxidant traits, we conducted a genome-wide association study with 404,628 single nucleotide polymorphism markers. Many significant associations were identified, including two homologs of Arabidopsis *(Arabidopsis thaliana) transparent testa* (*TT*) genes *TT10* and *TT4*. This study provides information that can help breeders incorporate health-promoting traits into elite breeding lines.

Polyphenols are a large class of phytonutrients that are beneficial to human health (Rice-Evans et al. 1996; Heinonen et al. 1998; Setchell and Cassidy 1999). They have the potential to protect against several chronic diseases, including cancer, type 2 diabetes, and heart disease (Del Rio et al. 2013). The mechanisms of action include anti-inflammatory (Terra et al. 2009), proapoptotic (Singh et al. 2009), hypolipidemic (Del Bas et al. 2009; Pajuelo et al. 2011), and antioxidant (Busserolles et al. 2006; Castrillejo et al. 2011) actions. Fruits are rich sources of polyphenols in the human diet, but polyphenols can also be found in some whole grain cereals, including wheat, rice, maize, and sorghum (Winkel-Shirley 2001; Himi and Noda 2005; Abdel-Aal et al. 2006; Liu 2007).

Sorghum is a major cereal crop grown worldwide for use as human food and livestock feed. Millions of people in sub-Saharan Africa and Asia subsist on food made from sorghum grain (FAO 1995). In the United States, sorghum is primarily used as animal feed but can be found in specialty food products (Janzen and Wilson 2002; Taylor et al. 2006; Elbehri 2007; Cureton and Fasano 2009). Some varieties of sorghum are rich sources of polyphenols, especially condensed tannins, which are high-molecular-weight polymers of catechins and epicatechins produced through the flavonoid pathway. The condensed tannins (hereafter referred to as tannins) are the only type of tannins that have been found in sorghum (Dykes and Rooney 2006). In sorghum seeds, tannins play a role in seed dormancy (Debeaujon et al. 2000) and protect against grain mold (Esele 1993) and bird predation (McMillian et al. 1972). Tannin-containing sorghum is reported to decrease feed efficiency in some animal species owing to tannin's ability to bind proteins and minerals (Santos-Buelga and Scalbert 2000); therefore, the majority of sorghum grown in the United States is nontannin (Awika and Rooney 2004). Conversely, many beneficial health effects are attributed to the polyphenols in sorghum, including antidiabetic (Farrar et al. 2008), antiobesogenic (Park et al. 2011), anticarcinogenic (Shih et al. 2007), and antioxidant (Dykes et al. 2005; Wu et al. 2011) effects.

The flavonoid pathway has been extensively studied in many plant species and is one of the most well understood biochemical pathways. In sorghum, two genes regulating the pathway have been cloned: *Yellow seed1*, which encodes a MYB protein with homology to the maize *P1* gene (Ibraheem et al. 2010), and *Tannin1*, which encodes a WD40 protein with homology to Arabidopsis *transparent testa glabra 1* (*TTG1*) (Wu et al. 2012). Rhodes et al. (2014) employed a genome-wide association study (GWAS) to identify the genetic controls of sorghum flavonoid variation and were able to identify *Tannin1* and *Yellow seed1*, as well as several novel candidate genes. The flavonoids were phenotyped with near-infrared spectroscopy (NIRS), a rapid, nondestructive method that predicts the quantitative value of a trait (Cozzolino 2014), but no sorghum polyphenol GWAS studies have been conducted using phenotype data collected through wet chemistry.

The antioxidant activity of some sorghum genotypes has been reported in a few studies; however, these studies were conducted on small sets with 10–50 samples (Awika et al. 2003; Dykes et al. 2005; Carbonneau et al. 2014) and restricted to a few commercial hybrids and advanced lines. The U.S. sorghum germplasm collection contains over 45,000 accessions representing landraces and cultivars from over 115 countries (USDA 2015), and the majority of the accessions have not been studied. The diverse material in the collection can be used to identify new germplasm and quantitative trait loci (QTLs) associated with antioxidant content, which could contribute to the development of molecular breeding tools for antioxidant-rich food-grade sorghum. The objectives of this study were 1) to find new antioxidant germplasm and 2) to identify QTLs associated with antioxidant activity, total polyphenols, and tannins using phenotype data generated through wet chemistry and a larger sorghum population than has been previously studied for antioxidants.

MATERIALS AND METHODS

Plant Material. A total of 266 sorghum accessions from the Sorghum Association Panel, representing domesticated sorghum from

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all five major races (bicolor, guinea, durra, kafir, and caudatum) (Casa et al. 2008) were investigated. Seeds were obtained from the U.S. National Plant Germplasm System's Germplasm Resources Information Network and planted in June 2014 in Manhattan, Kansas (USDA 2015). A complete random block design with two replications was used. Panicles were collected at physiological maturity in October 2014. Harvested panicles were air dried and mechanically threshed. To serve as a point of reference, fresh blueberries, a known source rich in phytonutrients and high antioxidant activity (Prior et al. 1998; Faria et al. 2005), were purchased from the local market and included for analysis.

Chemicals. (+)-Catechin, Folin–Ciocalteu reagent, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma-Aldrich (Saint Louis, MO, U.S.A.). Acetone, methanol, gallic acid, sodium phosphate dibasic, sodium phosphate monobasic, and sodium carbonate were purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.).

Extraction. The whole grain of each accession was ground into flour using a Udy cyclone sample mill (UDY, Fort Collins, CO, U.S.A.) with a 0.5 mm screen. All samples were milled using the same mill and procedure. Flour samples (0.3 g) were suspended in 10 mL of 75% (v/v) acetone and shaken for 2 h on a MaxQ 2500 shaker (Thermo Fisher Scientific). To enhance extraction, extracts (sample plus solvent) were stored overnight at –20°C, allowing for the phenolic compounds to completely diffuse. The extracts were centrifuged at 2,900 × *g* for 10 min and the supernatant decanted. The residues were reextracted with 10 mL of 75% acetone, shaken for 10 min, centrifuged at 2,900 × *g* for 10 min, and the supernatant added to the initial extract to obtain a total volume of 20 mL.

Tannin Determination. The tannin extraction method of Price et al. (1978) was used for sample preparation. Approximately 0.2 g of sorghum flour was extracted for 20 min in 8 mL of 1% concentrated HCl in methanol at 30°C in a water bath. The extracts were centrifuged at 805 × *g* for 4 min, and the supernatant was decanted for further testing. The high-throughput vanillin method of Herald et al. (2014) was followed to measure tannin concentration in the sorghum samples. Sorghum extracts and standards (30 µL) were pipetted into the appropriate wells of a 96-well plate. Vanillin reagent (150 µL) was added to the sample and standard wells simultaneously with a 96-tip pipettor (Sorenson Bioscience, Salt Lake City, UT, U.S.A.). After 1 min, 150 µL of 4% HCl was added to the sample control wells. The plates were incubated at 30°C for 20 min and read on a Synergy 2 microplate reader equipped with Gen5 data analysis software (Biotek Instruments, Winooski, VT, U.S.A.). All sample wells were read at the same time. Subsequently, all the sample control wells were read simultaneously. To keep the incubation and read times consistent, a 1 min time interval between reading the sample wells and the sample control wells was employed. Sample controls were used to avoid false positives. Methanol was used as a blank. The catechin standard curve was generated by using a concentration range of 0–1.2 mg/L. Tannin concentration was reported as catechin equivalents (g of CE/kg of sorghum flour). One method of categorizing sorghum is based on tannin concentration in the grain (Earp et al. 1981; Awika and Rooney 2004). Type 1 is considered a nontannin grain with <4 g of CE/kg; type 2 is considered a tannin-containing grain with 4–8 g of CE/kg; and type 3 is considered a tannin-containing grain with >8 g of CE/kg.

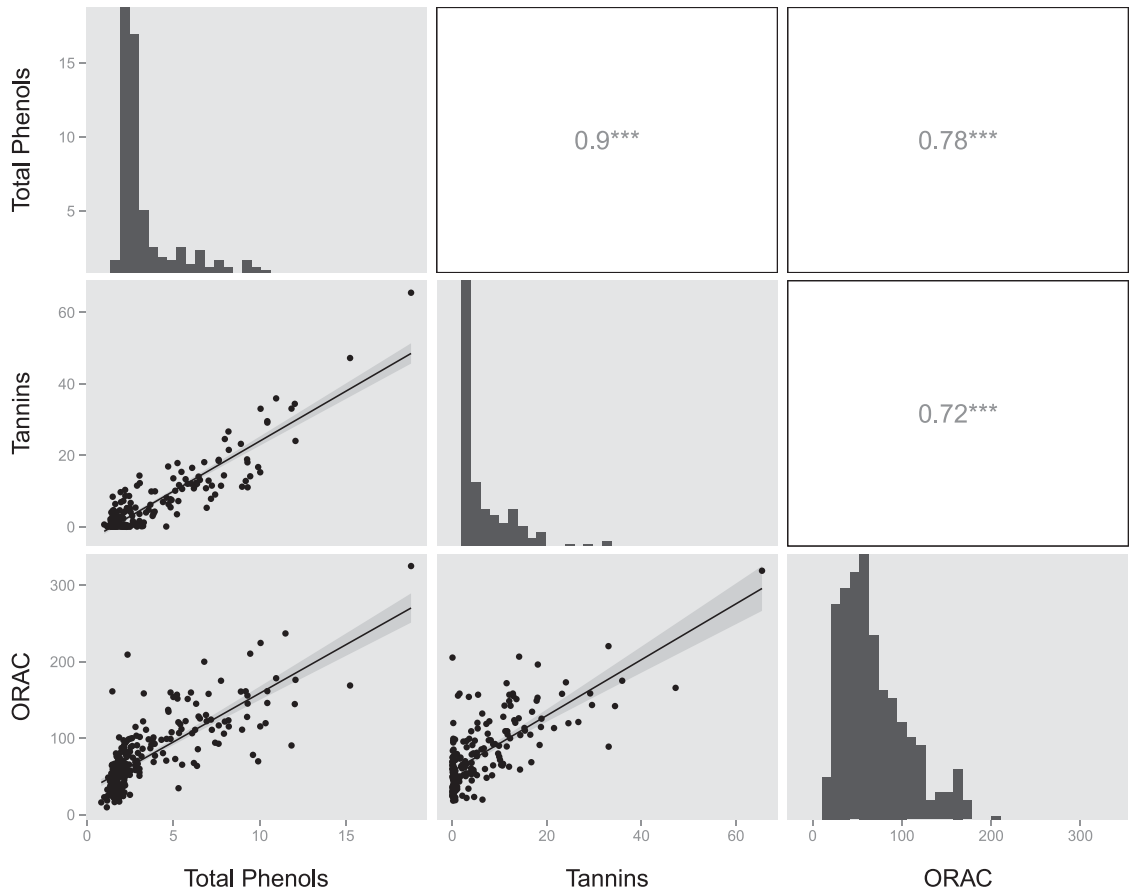

Fig. 1. Polyphenol, tannin, and oxygen radical absorbance capacity (ORAC) value relationships in the sorghum association panel. Scatter plots with regression lines showing the relationships between the traits are in the lower corner. Correlations between the traits are in the upper corner. Histograms of the mean concentrations of each trait are in the center diagonal. Units are g of GAE/kg for total polyphenols, g of CE/kg for tannins, and µmol of TE/g for ORAC, n = 266.

Total Phenolic Content. The method developed by Herald et al. (2012) was used to determine total polyphenols in the sorghum samples. Gallic acid (12.5–200 mg/L) was used as a standard to produce a calibration curve. Sample solutions without Folin–Ciocalteu reagent and Na_2CO_3 were used as negative controls. Acetone reagent was used as a blank. To each of the 96 wells, 75 µL of double distilled water was added followed by 25 µL of either the sample, control, standard, or blank. Next, 25 µL of Folin–Ciocalteu reagent (diluted 1:1 with double distilled water) was added to each well except the control wells. Excluding samples and standards, all were delivered through a repeating pipette. The solutions were mixed, and after 6 min 100 µL of 7.5% Na_2CO_3 was added to each well except the control wells. The solutions were mixed again, covered, and left in the dark for 90 min at ambient temperature. The absorbance was measured at 765 nm with a plate reader (set to shake for 60 s before reading). Each standard and sample solution was analyzed in triplicate. Total phenolic concentration was expressed as gallic acid equivalents (g of GAE/kg of sorghum flour).

Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay measures the in vitro peroxyl radical (ROO•, where R is an alkyl group) scavenging activity. The ORAC assay was conducted as described by Huang et al. (2002). Immediately before use a solution of 153mM AAPH in 75mM sodium phosphate buffer (pH 7.4) was prepared. A 4×10^{-3} mM fluorescein stock solution in 75mM sodium phosphate buffer (pH 7.4) was wrapped in foil and stored at 5°C. A working solution of diluted fluorescein (1:1,000) in 75mM sodium phosphate buffer (pH 7.4) was prepared immediately before use. The exterior wells of the 96-well plate were filled with 300 µL of deionized water. Fluorescein working solution (150 µL) was added to all inner wells. Solutions (25 µL) of diluted extract, phosphate buffer, and diluted trolox were added to samples, blank, and standard wells, respectively, for a total volume of 175 µL. The plate was equilibrated at 37°C for 30 min. All experimental wells were injected with 35 µL of AAPH solution using the plate reader injector and shaken for 10 s at maximum intensity. Fluorescence was monitored at 485 nm (excitation) and 528 nm (emission), with measurements taken from the top every 60 s for 1 h. A standard curve was generated (0–100 µM), and ORAC values were expressed in trolox equivalents (µmol of TE/g of flour). Based on the literature and the ORAC result in this study for blueberries, the measured ORAC values of the sorghum accessions were designated as low (<45 µmol of TE/g), high (45–150 µmol of TE/g), or rich (>150 µmol of TE/g) (Wu et al. 2004; Cho et al. 2005).

Genomic Analysis. The Sorghum Association Panel was previously genotyped (Morris et al. 2013a). We had genotypic data for 231 out of 266 phenotyped accessions. GWAS was carried out on 404,628 single nucleotide polymorphism (SNP) markers, using the statistical genetics package Genome Association and Prediction Integrated Tool (GAPIT) (Lipka et al. 2012). A standard mixed linear model (MLM) (Yu et al. 2006) with kinship (K), which controls for relatedness among the accessions in the panel, was performed (Zhang et al. 2010b). GAPIT corrected for multiple testing error using the Bonferroni correction (family-wise P value of 0.01, $P < 10^{-6}$). SNPs with minor allele frequencies (MAFs) ≥ 0.05 are reported. An a priori candidate gene list was previously developed (Morris et al. 2013b; Rhodes et al. 2014).

Statistical Analysis. The total measurements included two field replications × 266 lines × three different assays × triplicates on each assay × four subsamples per assay for a total of 19,080 for all the assays. Covariation was determined with Pearson's correlation coefficient. A simple linear regression was used to model the effect of tannins on polyphenols and antioxidant activity. P values of < 0.05 were considered statistically significant. Statistical analysis was conducted with R, a statistical computing language and environment (R Core Team 2015). Data obtained were expressed as mean ± standard deviation.

RESULTS

Phenotypic Variation in Polyphenols and Antioxidant Activity. We first investigated the variation of antioxidant activity, total polyphenols, and tannins and their covariation with each other. The ORAC values of the sorghum population ranged from 9.6 µmol of TE/g sorghum flour in PI597961 (SC1077) to 325.1 µmol of TE/g in PI534144 (SC84), with a mean of 74.4 µmol of TE/g (Fig. 1, Supplementary Table I). The data expressed a 34-fold difference in ORAC values within the population. The tannin accessions had significantly higher ORAC values (111.3 µmol of TE/g) than the nontannin accessions (57.6 µmol of TE/g; $P < 10^{-16}$). A total of

TABLE I
List of Accessions with Rich ORAC Activity (>150 µmol of TE/g) in Sorghum Grain Extracts[a]

Accession	Other Name	ORAC (µmol of TE/g)	Polyphenols (g of GAE/kg)	Tannins (g of CE/kg)	Grain Weight (mg)	Grain Color	Race	Origin
PI534144	SC84	325.1	18.8	65.5	19.2	Brown	Caudatum durra	Uganda
PI534117	**SC991**	**237**	**11.5**	**ND**	**23.1**	**Red**	**Bicolor**	**Uganda**
PI533902	*SC6*	*224.5*	*10.1*	*33*	*20.9*	*Brown*	*Bicolor durra*	*Ethiopia*
PI533833	SC319	201.5	9.45	14.15	28.4	Brown	Bicolor caudatum	Uganda
PI656063	**QL41**	**209.3**	**2.4**	**0.1**	**32.7**	**Red**	**Breeding line**	**Australia**
PI542718	San Chi San	200	6.8	18.1	36.8	Brown		China
PI533938	SC558	178.4	11.0	35.9	29.1	Brown	Caudatum	Zaire
PI533955	SC648	176.3	12.1	24	27.4	Brown	Caudatum kafir	South Africa
PI533752	SC103	175.1	7.8	11.5	31.9	Brown	Caudatum	South Africa
PI656102	SC59	168.9	15.2	47.2	20	Brown	Caudatum-bicolor	Sudan
PI656025	*Shan Qui Red*	*161.5*	*10.4*	*29.2*	*35.3*	*Brown*		*China*
PI533976	SC655	161.3	9.2	12.8	29.5	Brown	Caudatum	Sudan
PI533992	**SC575**	**161.2**	**1.5**	**1.6**		**White/tan**		
PI534155	SC155	160.9	8.9	23.2	24.7	Brown	Bicolor durra	Ethiopia
PI576434	SC1103	159.7	6	12.1	19	Brown		
PI533864	SC504	159.7	4.8	5.5	23.9	Red		
PI607931	**Tx2911**	**158.5**	**3.3**	**1.2**	**35.4**	**Red**	**Cultivar**	**U.S.A.**
PI656086	SC1471	157.9	7	12.9	34.1	Red		
PI656110	**SC968**	**156.9**	**5.2**	**3.5**	**20.7**	**White/tan**	**Bicolor durra**	**Zimbabwe**
PI276801	SC146	155.8	9.3	18		Brown		
PI576425	SC1155	153.9	5	13.6		Brown	Durra	Ethiopia
PI595740	SC1057	151.8	5.2	17.8	25.6	Yellow/red	Caudatum	Uganda
PI533957	*SC325*	*151.3*	*5.8*	*12.1*	*25.1*	*Brown*	*Caudatum*	*U.S.A.*

[a] Nontannin depicted in bold; 3-deoxyanthocyanidin-containing depicted with italics. ORAC = oxygen radical absorbance capacity; TE = trolox equivalents; GAE = gallic acid equivalents; CE = catechin equivalents; and ND = not detected.

23 accessions (5 nontannin and 18 tannin) possessed rich (>150 µmol of TE/g; Table I), 139 accessions (72 nontannin and 67 tannin) possessed high (45–150 µmol of TE/g), and 74 sorghum accessions (72 nontannin and 2 tannin) possessed low antioxidant activity (<45 µmol of TE/g). Approximately 98% of the tannin-containing sorghums had ORAC values greater than 45 µmol of TE/g. For comparison, the ORAC value for blueberries was 47.06 µmol of TE/g.

Total polyphenols in the 266 sorghum accessions ranged from 0.8 g of GAE/kg of sorghum flour in PI533866 (SC411) to 18.8 g of GAE/kg in PI534144 (SC84), with a mean of 3.4 g of GAE/kg (Fig. 1, Supplementary Table I). The data exhibited approximately a 24-fold difference in total polyphenols within the population. The population consisted of 179 (67%) nontannin accessions and 87 (33%) tannin accessions. The total polyphenols in the tannin-containing accessions ranged from 1.4 g of GAE/kg in PI533911 (SC58) to 18.8 g of GAE/kg in PI534144 (SC84), with a mean of 5.9 g of GAE/kg, whereas the nontannin accessions ranged from 0.8 g of GAE/kg in PI533866 (SC411) to 6.4 g of GAE/kg in PI595720 (SC1154), with a mean of 2.0 g of GAE/g. The blueberry total polyphenols value was 2.64 g of GAE/kg. Approximately 77% of the tannin and 12% of the nontannin sorghum accessions exhibited higher total polyphenol levels than the fresh blueberries.

Tannins ranged from nondetectable to 65.5 g of CE/kg of sorghum flour in PI534144 (SC84), with a mean of 4.8 g of CE/kg overall (Fig. 1, Supplementary Table I), a mean of 0.5 g of CE/kg in the nontannin accessions, and a mean of 13.4 g of CE/kg in the tannin-containing accessions. The data show a 66-fold difference in tannin concentration within the population.

ORAC, total polyphenols, and tannins were all significantly correlated with each other (Fig. 1). This agreed with previously reported data in smaller sample sets and confirmed that the trend held true in a larger and more diverse population. ORAC and total polyphenols were 78% ($P < 10^{-16}$), ORAC and tannins were 72% ($P < 10^{-16}$), and total polyphenols and tannins were 90% ($P < 10^{-16}$) correlated. We know that tannins are the major polyphenol in sorghum accessions that contain high concentrations of total polyphenols, so to determine how much variance in polyphenols could be explained by tannins, we fitted a linear model and found that tannins explained 82% of the variance in polyphenols ($P < 10^{-16}$). Because we expected that polyphenols contributed to the variance in ORAC values, we fitted a linear model to the data and found that total polyphenols explained 61% ($P < 10^{-16}$) and tannins explained 52% ($P < 10^{-16}$) of the variance in ORAC.

GWASs. *ORAC GWAS.* To investigate the genetic basis of variation in sorghum grain antioxidant activity, a GWAS was conducted. An MLM, which controls for population structure, identified 16 significant SNPs with MAFs ≥ 0.05 (Fig. 2A, Supplementary Table II). The most significant association peak colocalized with *Tannin1* (Sb04g031730) on chromosome 4 at ≈61 megabases (Mb). A significant SNP identified on chromosome 1 around 61.6 Mb (S1_61604263; $P < 10^{-9}$) was near *Yellow seed1* (Sb01g037670), which is required for 3-deoxyanthocyanidin production. GWAS also identified significant SNPs on chromosome 7 (S7_59361464; $P < 10^{-9}$) near a putative *TT4* homolog (Sb07g024260).

Because tannins explained 52% of the variance in ORAC values, GWAS was conducted using the residuals (an estimate of the amount of variation in ORAC that could not be explained by tannins) from the

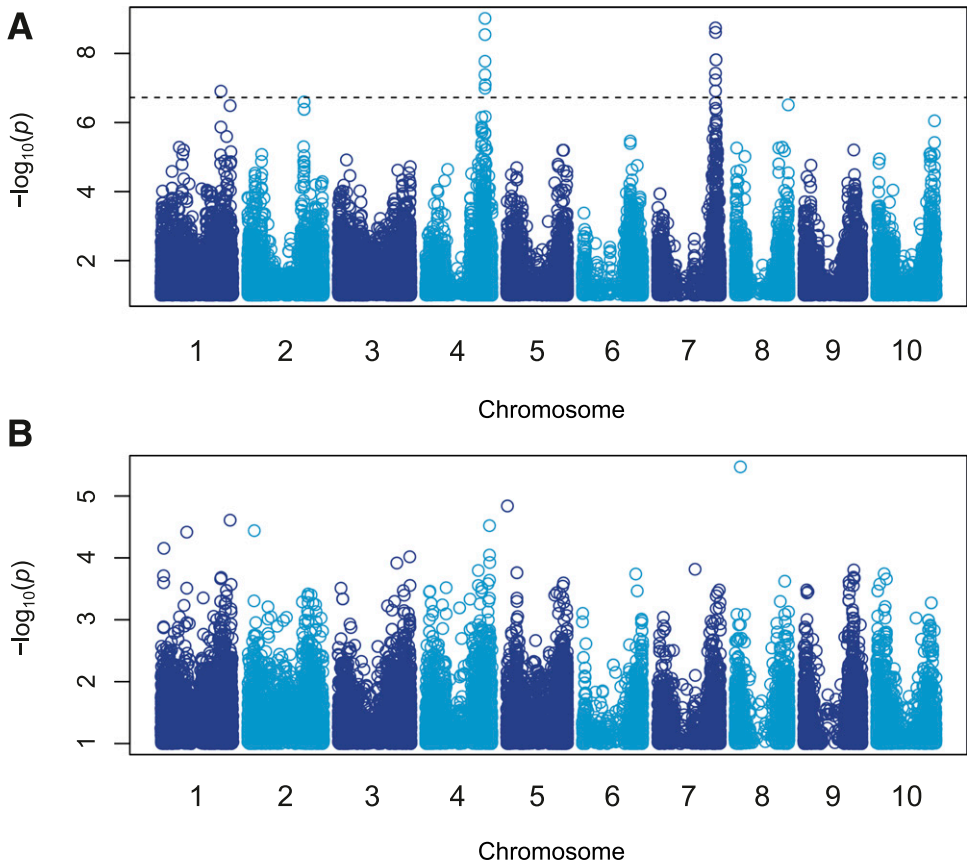

Fig. 2. Genome-wide association study (GWAS) for antioxidant activity of sorghum grain extracts. Scatter plot of GWAS results for oxygen radical absorbance capacity (ORAC) values (**A**) and ORAC values after controlling for tannin content (**B**). Negative log10-transformed *P* values (*y* axis) are plotted against the physical single nucleotide polymorphism (SNP) position on each chromosome (*x* axis). Each circle represents a SNP. The dashed horizontal line represents the genome-wide significance threshold as determined by Bonferroni correction. Regions with negative log10 *P* values above the threshold contain quantitative trait loci candidates.

linear model to identify nontannin genes controlling variance in ORAC values. An MLM did not identify any significant SNPs (Fig. 2B, Supplementary Table III), but the SNP with the lowest *P* value was on chromosome 8 at ≈5Mb (S8_5510878; $P < 10^{-6}$), near a putative *TT2* homolog (Sb08g004720).

Total Polyphenols GWAS. A GWAS was conducted to identify genetic controls of total polyphenol variation. An MLM identified 28 significant SNPs with MAFs ≥ 0.05 (Fig. 3A, Supplementary Table IV). As expected, the most significant SNP-trait associations colocalized with *Tannin1* on chromosome 4. Near the *Tannin1* association peak on chromosome 4 a significant SNP was identified at 57.7 Mb (S4_57786451; $P < 10^{-8}$) near a putative *TT10* homolog (Sb04g027850). A peak on chromosome 7 at ≈59 Mb (S7_59361464; $P < 10^{-9}$) was near the putative *TT4* homolog (Sb07g024260). A significant SNP on chromosome 8 at ≈54 Mb (S8_54549354; $P < 10^{-10}$) was near putative *TT8* (Sb08g022760) and *TT12* (Sb08g021640) homologs. GWAS also identified a significant SNP near *Yellow seed1* on chromosome 1. There was a significant association peak identified on chromosome 5 around 10 Mb (S5_10165709; $P < 10^{-11}$). There were no a priori gene candidates in this region. Also identified was a significant SNP at ≈7.5Mb on chromosome 2, near a putative *TT8* homolog (Sb02g006390) that has been implicated in tannin regulation in several studies (Nesi et al. 2000; Furukawa et al. 2007; Mace and Jordan 2010; Wu et al. 2012; Morris et al. 2013b).

Because tannins explained 82% of all the variance in total polyphenols, it was expected that most of the significant SNPs identified in the GWAS would be near known tannin genes. To identify nontannin genes that may be contributing to total polyphenol variance, we conducted a GWAS using the residuals from the linear model. The MLM (Fig. 3B, Supplementary Table V) identified one significant SNP on chromosome 2 at ≈62 Mb (S2_61994553; $P < 10^{-16}$) near a putative *bronze2* (*Bz2*) maize homolog (Sb02g027080).

Tannins GWAS. A GWAS was conducted to identify alleles associated with quantitative variation of tannins. An MLM identified 17 significant SNPs with MAFs ≥ 0.05 (Fig. 4, Supplementary Table VI). As expected, the most significant association peak colocalized with *Tannin1* at ≈61 Mb. As with the total polyphenols GWAS, there was a significant association peak identified on chromosome 5 around 10 Mb (S5_10165709; $P < 10^{-11}$) with no a priori gene candidates nearby, on chromosome 4 at 57.7 Mb (S4_57786451; $P < 10^{-8}$) near a putative *TT10* homolog (Sb04g027850), and on chromosome 7 (S7_59361464; $P < 10^{-9}$) near a putative *TT4* homolog (Sb07g024260). Another significant SNP was identified on chromosome 3 at ≈8 Mb (S3_8329045) near an a priori candidate that is a putative homolog to the grape *leucoanthocyanidin reductase1* (*VvLAR*) gene (Sb03g008760).

Comparison Between Environments. To better understand the environmental contribution to sorghum grain polyphenols, we compared tannin GWAS results from the Sorghum Association Panel grown in Manhattan, Kansas (referred to as KS) to tannin GWAS results from the Sorghum Association Panel grown in Florence, South Carolina (referred to as SC) in 2012 (Rhodes et al. 2014). Pearson's correlation was calculated to assess the relationship of tannins between SC and KS. A significant correlation was found between tannins in each panel ($r = 0.59$, $P < 10^{-15}$). The range of tannins in the SC panel was nondetectable to 78.5 g of CE/kg with a mean of 10.4 g of CE/kg, compared with the KS panel, which ranged from nondetectable to 65.5 g of CE/kg, with a mean of 4.8 g of CE/kg. Next, we compared the significant SNP associations between the two panels. GWAS conducted on the SC panel identified 21 significant SNPs with MAFs ≥ 0.05, compared with 17 significant SNPs with MAFs ≥ 0.05 in the KS panel. In both panels, the tannin GWAS identified a major association peak on chromosome 4 (≈61 Mb) near *Tannin1*, as well as significant SNPs on chromosome 5 (≈8–12 Kb) with no a priori candidates nearby. In the KS panel, but not the SC panel, the tannin GWAS identified significant associations with six SNPs: S7_59361562 (near a *TT4* homolog), S9_52671750 (near a *TTG2* homolog), S4_57657307

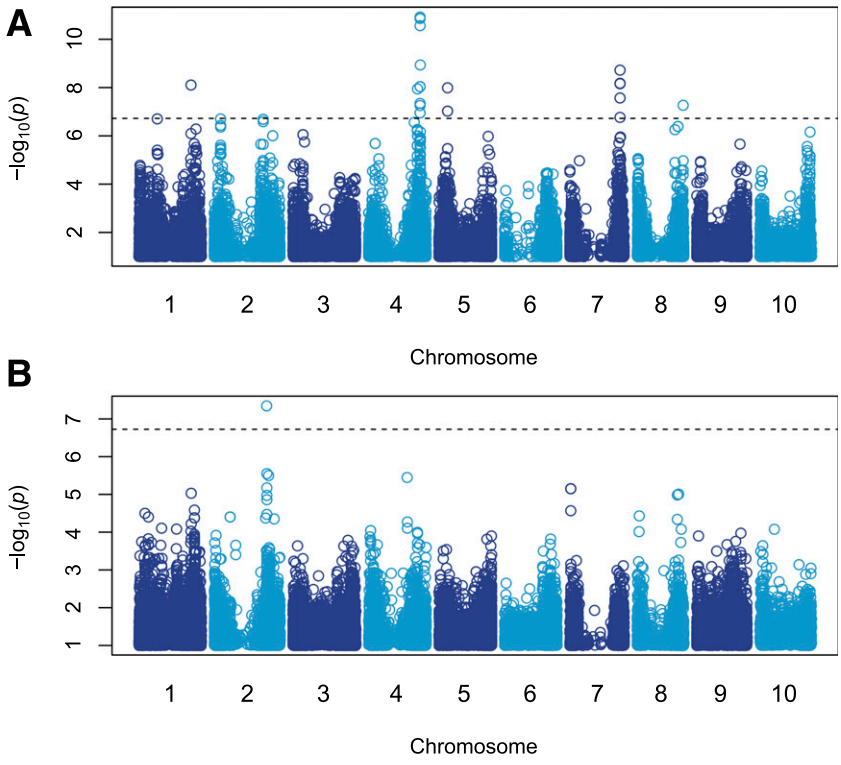

Fig. 3. Genome-wide association study (GWAS) for total polyphenols in sorghum grain. Scatter plot of GWAS results for total polyphenols (**A**) and total polyphenols after controlling for tannin content (**B**). Negative log10-transformed *P* values (*y* axis) are plotted against the physical single nucleotide polymorphism (SNP) position on each chromosome (*x* axis). Each circle represents a SNP. The dashed horizontal line represents the genome-wide significance threshold as determined by Bonferroni correction. Regions with negative log10 *P* values above the threshold contain quantitative trait loci candidates.

(near a *TT10* homolog), S3_8329045 (near a *VvLAR1* homolog), S2_57798835 (near a *TT7* homolog), and S4_50511842 (near a *TT1* homolog). In the SC panel but not the KS panel, the tannin GWAS identified significant associations with five SNPs: S1_53978857 (near a *BZ2* homolog), S3_59791550 (near a *BZ2* homolog), S1_4155554 (near a *TT5* homolog), S4_59070803 (near a *VvLAR1* homolog), and S4_62914607 (near a *TT1* homolog).

DISCUSSION

Crop Improvement of Polyphenols and Antioxidants in Sorghum Grain. Efforts to improve the nutritional quality of food crops through classical breeding have led to many nutritionally improved varieties in crops, such as carrots with increased provitamin A carotenoids (Simon et al. 1989; Just et al. 2007) and corn with lysine and tryptophan (Gibbon and Larkins 2005). Our diverse sorghum population contained a large range of antioxidant activity, total polyphenols, and tannins, which may help guide breeders in selecting accessions to develop health-promoting specialty varieties and hybrids. Sorghum has ORAC values that are comparable to or higher than other grains that have been reported in the literature—colored rice bran with 535–1,876 µmol of TE/g and charcoal purple wheat with 80 µmol of TE/g (Liu et al. 2010; Zhang et al. 2010a; Min et al. 2011). Many sorghum accessions in this study had higher ORAC values than the fresh blueberries; thus, it is reasonable to assume that many of the sorghum accessions can serve as rich

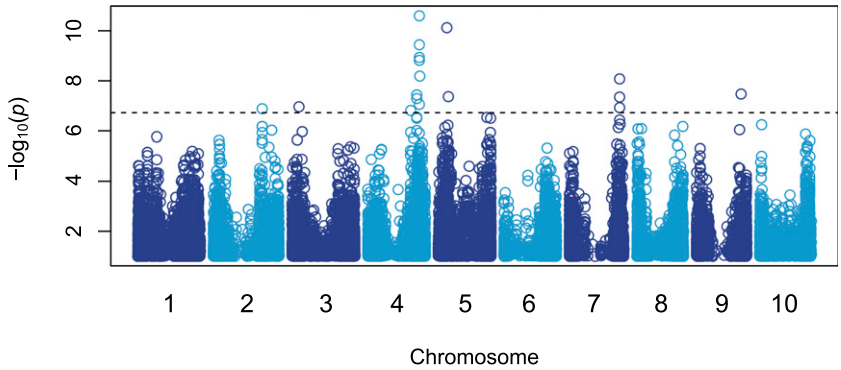

Fig. 4. Genome-wide association study (GWAS) for tannin concentration in sorghum grain. Scatter plot of GWAS results for tannin. Negative log10-transformed *P* values (*y* axis) are plotted against the physical single nucleotide polymorphism (SNP) position on each chromosome (*x* axis). Each circle represents a SNP. The dashed horizontal line represents the genome-wide significance threshold as determined by Bonferroni correction. Regions with negative log10 *P* values above the threshold contain quantitative trait loci candidates.

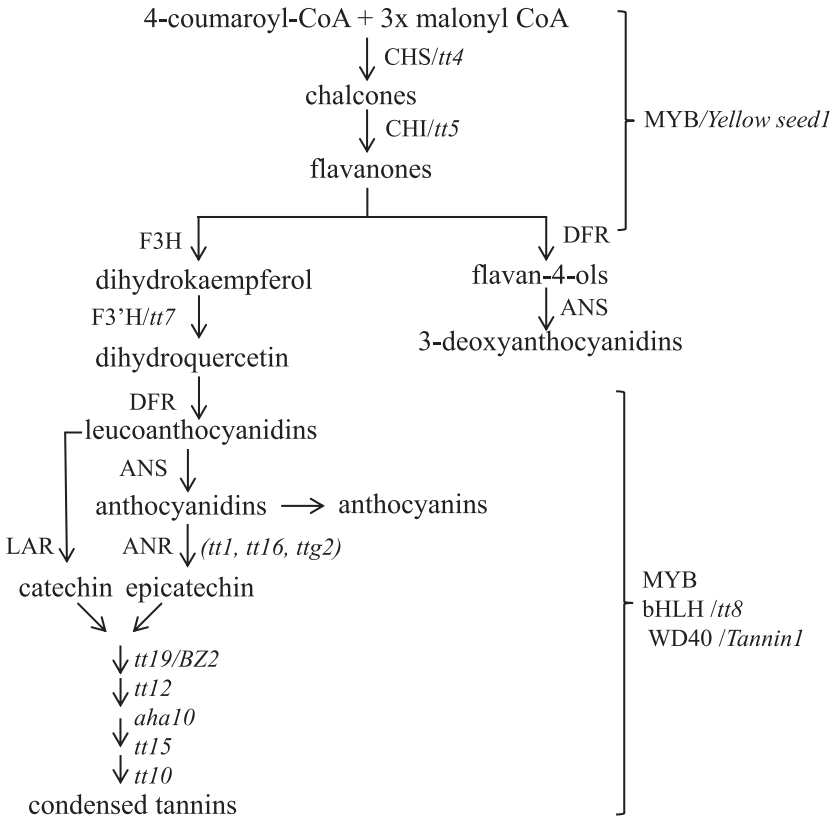

Fig. 5. Flavonoid biosynthetic pathway. Enzymes are represented with uppercase letters, and genes are represented with italicized letters. CHS = chalcone synthase; F3H = chalcone-flavanone isomerase hydroxylase; F3′H = flavanone 3′-hydroxylase; DFR = dihydroflavonol-4-reductase; ANS = anthocyanidin synthase; ANR = anthocyanidin reductase; and LAR = leucoanthocyanidin reductase.

sources of dietary antioxidants. Awika et al. (2003) reported sorghum grain ORAC values of 140 and 868 μmol of TE/g for nontannin red and tannin-containing sumac, respectively. Kaufman et al. (2013) reported sorghum bran ORAC values as high as 1,100 μmol of TE/g. Phenolic compounds are concentrated in the bran of sorghum grain, so higher ORAC values are reported in the studies that used bran compared with the current study, which used whole grain flour.

In general, our results showed trends similar to those cited in the literature on smaller, less diverse sample sets, indicating that accessions with higher tannin concentrations are higher in total polyphenols and antioxidant activity (Hagerman et al. 1998; Tian et al. 2012). It is interesting, though, that not all the accessions in our study followed this pattern, because there were nontannin lines such as PI534117 (SC991) and PI656063 (QL41) that exhibited higher ORAC values than some tannin-containing accessions. This leads one to speculate that there are additional unidentified compounds contributing to the variation in antioxidant activity. Additional diverse panels, such as the sorghum mini core collection (Upadhyaya et al. 2009) and the Generation Challenge Program sorghum reference set (ICRISAT n.d.), will aid in the discovery of new sorghum germplasm with high antioxidant activity.

We identified 23 sorghum accessions with especially high ORAC values. Of particular interest, five of the high-ORAC accessions were nontannin sorghums (PI534117 [SC991], PI656063 [QL41], PI533992 [SC575], PI607931 [Tx2911], and PI656110 [SC968]) and three of the high-ORAC accessions were tannin-containing sorghums that produce 3-deoxyanthocyanidins (PI533902 [SC6], PI656025 [Shan Qui Red], and PI533957 [SC325]) (Rhodes et al. 2014). The 3-deoxyanthocyanidins are phytoalexins that may play a protective role against chronic inflammatory diseases and cancer (Awika et al. 2005). Also of interest, PI613536 (KS115) is a yellow-grained high-ORAC tannin breeding line that produces carotenoids (Salas Fernandez et al. 2009), compounds that are precursors to vitamin A and are necessary for many immune functions (Rao and Rao 2007).

Genetic Controls of Polyphenols and Antioxidants in Sorghum. Several significant SNP associations were identified near putative homologs of known flavonoid pathway genes in other plant species (Fig. 5), some of which were not identified in the previous polyphenol GWAS study by Rhodes et al. (2014), including *VvLAR*, *Bz*2, and *TT1* homologs. Differences in accessions between the two locations may contribute to the differing significant SNPs identified in each panel. The SC panel contained additional tannin-containing accessions that were not in the KS panel, which may have contained unique alleles for high tannin. It is also possible that environmental differences (KS versus SC) or measurement differences (chemistry for KS samples versus NIRS for SC samples) contributed to the differences in QTLs identified. Transcriptional regulation of flavonoid pathway genes can be modulated by many environmental factors—including abiotic factors such as temperature or rainfall and biotic factors such as fungal or insect attack—so it is not surprising that there are differences in QTLs between environments (Xu et al. 2015).

The significant SNPs identified on chromosome 4 near *Tannin1* (≈60.8–61.1 Kb) were also identified in a GWAS conducted on a subset of the Florence, SC, samples that were all tannin-containing accessions (Rhodes et al. 2014). It is worth speculating that the SNPs near *Tannin1* represent true associations with one or more genes other than *Tannin1,* because quantitative tannin variation in the subset should not be regulated by *Tannin1*, as all accessions in this subset carry the wild-type *Tannin1* allele. It is possible that one of the a priori candidate genes near *Tannin1*—for example, Sb04g030570 (*leucoanthocyanidin reductase 1*), Sb04g030840 (*TTG1*), or Sb04g031110 (*Zm1/TT2)*—is involved in regulating quantitative variation in tannin concentration. A possible a posteriori gene candidate is a nearby putative gene encoding a class III peroxidase (Sb04g031120), an enzyme that may be involved in oxidation or condensation reactions in the final steps of the tannin pathway (Pourcel et al. 2007; Zhao et al. 2010).

One previously unidentified SNP significantly associated with total polyphenols was identified by controlling for tannins (by using the residuals from the linear model) in the GWAS. The total polyphenols GWAS identified a putative homolog of *BZ2*, which is a glutathione *S*-transferase gene in maize required for anthocyanin transport into the vacuoles (Marrs et al. 1995). Although the ORAC GWAS using residuals did not identify any significant SNPs, the SNP with the lowest *P* value was near a putative *TT2* homolog, which is an Arabidopsis MYB gene involved in regulation at many points in the flavonoid pathway (Hichri et al. 2011). The sample size of 231 accessions may have been too small to detect small-effect or rare allelic variants in the ORAC GWAS using residuals. Future studies could improve statistical power by increasing the sample size or by using biparental linkage mapping or nested association mapping to dissect the genetic controls underlying phenotypic variation of antioxidant traits (Myles et al. 2009).

Of interest, the total polyphenols and ORAC GWAS using residuals did not have top SNPs in common, suggesting there are compounds unrelated to polyphenol variation that underlie ORAC variation after accounting for the effects of tannins. Many other compounds in grain contribute to antioxidant activity, including vitamin E and carotenoids (Fardet et al. 2008), and future studies characterizing these compounds in sorghum grain may help determine the genes underlying the SNPs identified in the ORAC GWAS.

CONCLUSIONS

Overall, this research cataloged both tannin and nontannin accessions with high antioxidant activity and identified several QTLs for tannin variation that had not been previously identified. Moreover, QTLs for variation in sorghum grain antioxidant activity were identified for the first time. The high-antioxidant accessions and genetic markers will contribute to efforts aimed at developing specialty sorghum with human health benefits.

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