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Occurrence of the Waxy Alleles *wx*^{a} and *wx*^{b} in Waxy Sorghum Plant Introductions and Their Effect on Starch Thermal Properties

J. F. Pedersen,★ R. A. Graybosch, and D. L. Funnell

ABSTRACT

The existence of two waxy alleles, *wx*^{a} associated with no detectable granule bound starch synthase (GBSS) and *wx*^{b} associated with apparently inactive GBSS, was recently reported in sorghum [*Sorghum bicolor* (L.) Moench]. In this paper, the occurrence of the *wx*^{a} and *wx*^{b} alleles in the USDA-ARS photoperiod-insensitive sorghum collection was determined, and the effects of the *wx*^{a} and *wx*^{b} alleles on thermal properties of sorghum starch (gelatinization temperatures and energy requirements) measured by differential scanning calorimetry. Of the 51 purported waxy accessions examined, 14 tested positive for presence of amylose by iodine staining and were considered to be previously misclassified wild-type lines. Nine accessions were mixed for presence or absence of amylose. Twenty-four of the 28 accessions confirmed to be waxy by negative iodine staining for amylose had no detectable GBSS using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (*wx*^{a}), and four were show to contain GBSS (*wx*^{b}). Mean gelatinization onset, peak, and end temperatures were significantly lower for wild-type than either of the two waxy genotypes. Mean gelatinization onset temperature was slightly higher for waxy-GBSS+ genotypes than waxy-GBSS– genotypes. Mean gelatinization end temperature was slightly higher for waxy-GBSS– genotypes than waxy-GBSS+ genotypes. Significant genetic variation was observed within genotypic classes, suggesting influence of additional modifier genes affecting sorghum starch structure or micro-environmental effects.

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Abbreviations: DSC, differential scanning calorimeter; GBSS, granule bound starch synthase; PI, plant introduction; QTL, quantitative trait locus; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Sorghums [*Sorghum bicolor* (L.) Moench] with modified starch are receiving increased attention as the sorghum industry explores new food and industrial uses for its grain. The waxy phenotype has been recognized in sorghum since 1933 (Karper, 1933) and is associated with endosperm starch lacking amylose and composed of nearly 100% amylopectin. In cereals, this condition is usually due to a lack of functionality of the amylose–forming enzyme granule bound starch synthase (GBSS; EC 2.4.1.242).

Presence or absence of amylose has significant impact on the physicochemical properties of starch. Amylose can form a firm gel, while amylopectin exhibits low syneresis and high resistance to retrogradation (Takahiro et al., 2003). Amylose molecules tend to predominate in amorphous regions of starch granules. Crystalline regions are dominated by amylopectin, with the degree of crystallinity thought to be a function of the branch frequency, and branch length, of amylopectin (Martin and Smith, 1995). However, differences in physicochemical properties of starches from waxy vs. wild-type grains at the species level have been documented. Jane

et al. (1999) reported starches from waxy rice (*Oryza sativa* L.) with low retrogradation rates compared to starch from wild-type rice, while retrogradation rates of starch from waxy maize (*Zea mays* L.) were similar to starch from wild-type maize. More recent research on rice has shown physicochemical differences among low amylose lines within the species (Takahiro et al., 2003).

As in many other cereals, sorghum was recently shown to have variants at the waxy loci. In a set of eight waxy sorghum lines, Pedersen et al. (2005) discovered the existence of two waxy alleles, wx^a and wx^b. Waxy lines with allele wx^a produce no detectable GBSS. In waxy lines carrying the waxy allele wx^b, GBSS is produced, detectable by both gel electrophoresis and immunoblotting, but it is evidently nonfunctional. With respect to GBSS production, the allele wx^a is recessive to wx^b, and, and with respect to amylose synthesis, both waxy alleles are recessive to the wild-type, *Wx*. Only two of the eight lines had the wx^b allele, and both were reported to share a common pedigree (Pedersen et al., 2005). The frequency and distribution amongst sorghum germplasm of the two *wx* alleles, and their effects on starch thermal properties, is unknown.

One method of describing physicochemical and thermal properties of starches is the differential scanning calorimeter (DSC). This method has become "the method of choice for studying starch gelatinization phenomena" and measures the difference in enthalpy change that occurs during gelatinization of starch samples (Lund, 1984, p. 254). These gelatinization phenomena have impact on cooking, textural properties, digestibility, and an array of other characteristics impacting the food industry. Numerous studies, especially with maize starch, have employed DSC as a means of assessing both genetic and environmental factors conditioning starch variation. White et al. (1990) detected significant variation in DSC gelatinization onset and peak temperatures, both within and across open pollinated maize populations. Sanders et al. (1990) used DSC to characterize maize starches derived from lines carrying one or more of the endosperm mutants *wx*, *a*, *su*, and *du*. Differential scanning calorimeter onset and peak gelatinization temperatures of the mutants and of lines carrying various combinations of mutations differed from each other and from wild-type. Scott and Duvick (2005) studied the distribution of quantitative trait loci (QTLs) controlling starch variation, as detected by DSC. They found one such QTL to cosegregate with the maize *Wx1* locus, known to control GBSS synthesis.

Relative to maize, comparatively little is known of genetic variation for starch properties in sorghum. The USDA-ARS photoperiod-insensitive sorghum germplasm collection (http://www.ars-grin.gov/npgs/) contains 53 accessions that have been identified as waxy. The following study was therefore designed to determine the occurrence of the wx^a and wx^b alleles in this genetically diverse set of sorghum lines, to determine the effects of the wx^a and wx^b alleles on thermal properties of sorghum starch as measured by DSC, and to determine whether genetic variation exists in properties of sorghum starch within wx^a and wx^b genotypic groups.

MATERIALS AND METHODS

Fifty-three Plant Introductions (PIs) identified as waxy from the USDA-ARS photoperiod-insensitive sorghum collection were obtained from the USDA-ARS Southern Regional Plant Introduction Station, Griffin, GA (http://www.ars-grin.gov/npgs/). Accession number, local name, and country of origin are shown in Table 1. Seed quantities of 51 of these lines (two did not set seed before frost) were increased by self-pollination under bags in 2002. PI 562768 produced a mixture of red and white seed. PI 455543 produced a mixture of white and brown seed, but only the white seed reached maturity.

The 51 accessions; 'Ellis', a waxy control; and nine wild-type controls ('Atlas', 'Brawley', 'Dale', E35-1, IS2261, 'Kansas Collier', N98, 'Rox Orange', and 'Wray') were planted in replicated field trials in 2003 and 2004 at the University of Nebraska Field Laboratory, Ithaca, NE (Sharpsburg silty clay loam; fine, smectitic, mesic Typic Argiudoll). Plots consisted of single 7.6-m rows spaced 76 cm apart. Each plot was seeded with a precision vacuum planter calibrated to deliver 120 seeds per row (240,000 seeds ha^{-1}). The experiments were planted 22 May 2003 and 21 May 2004. Nitrogen fertilizer was applied preplant at 157 kg ha^{-1}. Atrazine was applied at 2.2 kg ha^{-1} immediately after planting, followed by an application of quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) and atrazine at 0.37 kg ha^{-1} and 1.1 kg ha^{-1}, respectively approximately 14 d postemergence. Grasshoppers [*Dissosteira carolina* (Linnaeus)] were controlled by application of chloropyrifos [phosphorodithioic acid, O,O-diethyl *O*-(3,5,6-trichloro-2-pyridyl) ester] on 17 July 2003. In 2003 2.5 cm of supplemental irrigation was applied via overhead sprinklers on 24 July, 14 August, and 28 August, and 5 cm of supplemental irrigation was applied on 4 August and 7 August. In 2004 2.5 cm of supplemental irrigation was applied on 3, 12, and 19 August. Ten panicles per plot were bagged before anthesis to prevent outcrossing, hand-harvested on reaching maturity, threshed, and the grain stored at 7°C until laboratory analyses. The experimental design was a randomized complete block with four replications in each of the 2 yr.

Presence or absence of amylose was determined by staining with iodine as described in Pedersen et al. (2004). Presence or absence of the GBSS protein was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in Gaines et al. (2000). For amylose and GBSS classification, grain from two replicates from 2003 was tested. Entries were grouped into the following phenotypes: wild-type, waxy-GBSS+, waxy-GBSS−. Entries comprised of a mixture of wild-type and waxy grains were excluded from further analyses.

Starch Extraction

Three grams of seed per plot was cleaned by hand to remove broken seeds and foreign material, and then steeped in 10 mL of 1% sodium metabisulfite for 24 h at 45°C (Ji et al., 2004). The

Table 1. Accession number, local name, and country of origin of lines previously reported to be waxy and photoperiod insensitive in the USDA-ARS sorghum germplasm collection, control varieties, and results of iodine test for amylose and sodium dodecyl sulfate polyacrylamide gel electrophoresis test for granule bound starch synthase (GBSS).

Accession no.	Local name	Country of origin	Amylose present	GBSS present	Allele
PI 23231	Brown Kaoliang	China	no	yes	wx^b
PI 55123	Hemaise	Sudan	no	no	wx^a
PI 76407	North West Gold Kaoliang	China	no	no	wx^a
PI 82340	Kaoliang-WX	Korea	no	no	wx^a
PI 87355	Bomususu	Korea	no	no	wx^a
PI 88004	Susu zairai shu	Korea	no	no	wx^a
PI 173971	Jawar	India	yes	yes	Wx
PI 175316	Mal Giunra	India	yes	yes	Wx
PI 192876	Katengu	Indonesia	no	no	wx^a
PI 217896	244	Indonesia	mixed	yes	?
PI 217897	305	Indonesia	no	yes	wx^b
PI 220636	Nai-Shaker	Afghanistan	no	no	wx^a
PI 234456	Unknown local name	Japan	no	no	wx^a
PI 246699	IS 1024	India	yes	yes	Wx
PI 250230	MN 4116	Pakistan	yes	yes	Wx
PI 455538	ETS 3632	Ethiopia	mixed	yes	?
PI 455543	ETS 3634	Ethiopia	no	no	wx^a
PI 547915	Bai Ruan Gao Liang	China	yes	yes	Wx
PI 548008	Huang Ke Jiao	China	no	no	wx^a
PI 562758	Basuto Red Q2-1-29	USA	no	no	wx^a
PI 562768	Purdue 81540	USA	mixed	yes	?
PI 563015	Kaura Mai Faran Kona	Nigeria	no	no	wx^a
PI 563068	IS 8303	USA	no	no	wx^a
PI 563395	IS 10464	Uganda	mixed	yes	?
PI 563402	IS 10497	USA	no	no	wx^a
PI 563576	LV 129	China	no	no	wx^a
PI 563611	LR 390	China	yes	yes	Wx
PI 563612	LR 395	China	yes	yes	Wx
PI 563670	L 1999B-17	China	no	yes	wx^b
PI 563671	L 1999B-18	China	no	yes	wx^b
PI 563672	LR 2409	China	yes	yes	Wx
PI 565116	SDS 1412	Zimbabwe	yes	yes	Wx
PI 567796	Pyungchang local	South Korea	no	no	wx^a
PI 567799	Pyungchang local	South Korea	mixed	yes	?
PI 567803	Yungju local	South Korea	no	no	wx^a
PI 567809	Unknown local name	South Korea	no	no	wx^a
PI 567810	Unknown local name	South Korea	mixed	no	?
PI 567811	Unknown local name	South Korea	no	no	wx^a
PI 567910	Bai Liu Zi (Fu Yang)	China	yes	yes	Wx
PI 567913	Bai She Yan (Sui Zhong)	China	yes	yes	Wx
PI 567931	Da Shan Dong (Wen Shui)	China	yes	yes	Wx
PI 567939	Gao Liang	China	yes	yes	Wx
PI 567965	Da E Huang	China	mixed	yes	
PI 568012	Niu Xin	China	yes	yes	Wx
PI 568031	Da Luo Chui	China	mixed	yes	?
PI 585348	IS 24522	Lebanon	no seed	no seed	?
PI 586448	Cody	Hungary	no	no	wx^a
PI 586454	Leoti	Hungary	no	no	wx^a
PI 586524	IS 27929	China	no	no	wx^a
PI 586526	IS 27931	China	no	no	wx^a
PI 586529	IS 27935	China	no	no	wx^a

Table 1. Continued.

Accession no.	Local name	Country of origin	Amylose present	GBSS present	Allele
PI 586532	IS 27938	China	mixed	no	?
PI 591372	IS 3244	India	no seed	no seed	?
		Waxy control			
	Ellis	USA	no	no	*wx*[a]
		Wild-type controls			
NSL 3986	Atlas	USA	yes	yes	*Wx*
NSL 4346	Brawley	USA	yes	yes	*Wx*
NSL 7433	Dale	USA	yes	yes	*Wx*
–	E35-1	USA	yes	yes	*Wx*
–	IS 2261	USA	yes	yes	*Wx*
–	Kansas Collier	USA	yes	yes	*Wx*
PI 535783	N98	USA	yes	yes	*Wx*
–	Rox Orange	USA	yes	yes	*Wx*
NSL 117772	Wray	USA	yes	yes	*Wx*

seeds were then homogenized in 50 mL distilled water using a single speed Waring blender (Waring Laboratory, Torrington, CT) and a 110-mL minicontainer. This slurry was then filtered through four layers of cheesecloth and allowed to settle at 4°C for 1 h. The starch was then layered onto 10 mL of 60% cesium chloride and centrifuged at $1643 \times g$ using a Beckman GS-6 swinging bucket centrifuge (Beckman Coulter Inc., Fullerton, CA) to separate the starch from impurities. The starch was then washed three times with 10 mL of water and allowed to dry overnight at 34°C (Beta et al., 2000). The dried starch was then placed in a moisture-controlled cabinet for 4 d. Moisture content was determined by heating a portion of the sample to 140°C for 30 min and calculating the difference in mass before and after heating.

Thermal Analyses

Analyses of the starch gelatinization were made in triplicate for each sample using a Pyris Diamond DSC and Pyris v. 7 software (PerkinElmer, Norwalk, CT). Approximately 10 mg dry starch for each sample was placed inside a high-pressure stainless steel pan (PN: 03190029, PerkinElmer, Norwalk, CT) along with 55 ∝l autoclaved water to give a starch/water ratio of 1:5 (Sichina, 2000). The sample pans were then sealed and stored at room temperature overnight to allow starch to hydrate. During the experiment, samples were allowed to equilibrate at 40°C for 2 min before being heated to 120°C at 10°C min^{-1}. The DSC was calibrated with indium and an empty pan was used as a reference (Iturriaga et al., 2004). Pyris software (v. 7, PerkinElmer) was used to calculate gelatinization transition temperatures (onset, peak, and end), total heat of transition or

enthalpy, and the amount of heat required to initiate gelatinization (peak height).

Statistical Analyses

To contain costs, only two replications per year were initially analyzed using the DSC. After examination of the resulting data, the decision was made to limit analyses using the DSC to those two replications. The DSC data analysis for this paper was generated using the PROC MIXED procedure of SAS (SAS Institute, 2002–2003) with years and reps considered random. Phenotype and entries within phenotype were considered fixed. All reported means were generated using the LSMEANS function, and significance of differences between phenotypes was determined using a *t* test, calculated by the DIFF function of PROC MIXED.

RESULTS

Of the 51 purported waxy accessions examined, 14 tested positive for presence of amylose and for presence of GBSS (Table 1). These accessions were considered to be wild-type lines misclassified by previous investigators as waxy. Another nine accessions were mixed for presence or absence of amylose-containing grain. Of the nine accessions mixed for the presence or absence of amylose-containing grain, one accession, PI 567810, tested negative for GBSS. Upon examination of the raw data, only one seed of 18 tested positive for amylose leading to speculation that a false positive or mechanical seed mixture may have been observed for amylose in PI 567810. Four of the 28 accessions confirmed to be waxy by negative iodine staining for amylose were shown to contain GBSS.

Mean gelatinization onset, peak, and end temperatures were significantly ($P = 0.05$) lower for wild-type than either of the two waxy genotypes (Table 2). Mean gelatinization onset temperature was slightly higher ($P = 0.05$) for waxy-GBSS+ genotypes than waxy-GBSS– genotypes, while mean gelatinization end

Table 2. Least square means of differential scanning calorimeter data for waxy-GBSS+, waxy-GBSS−, and wild-type sorghum grain.

	Onset temperature	Peak temperature	End temperature	Enthalpy (ΔH)	Peak height
		°C		J g^{-1}	
Waxy/GBSS−	69.2b[†]	74.7a	80.8a	16.4a	4.17b
Waxy/GBSS+	70.1a	74.7a	80.3b	16.9a	4.71a
Wild-type	66.8c	72.0b	77.6c	15.3b	4.05c

[†]Means in a column followed by the same letter are not significantly different at $P = 0.05$.

temperature was slightly higher (P = 0.05) for waxy-GBSS− genotypes than waxy-GBSS+ genotypes. Mean gelatinization peak temperature and mean enthalpy were equivalent (P = 0.05) for waxy-GBSS− genotypes and waxy-GBSS+ genotypes. For all five DSC traits measured, when examining individual accessions considerable variation was observed within each genotype group (Fig. 1–5).

DISCUSSION

Within this group of 51 purported waxy accessions, 27% were wholly misclassified and 17% are previously undetected mixtures. It is our understanding that identification of waxy accessions in the U.S. sorghum collection has been accomplished primarily using visual examination of grain fracture patterns. Clearly, simple chemical tests such as iodine staining (Pedersen et al., 2004) are more reliable. Our experience in working with waxy sorghum also leads us to conclude that the inverse error, failure to identify lines with pure amylopectin, or waxy lines, can easily occur. Although not investigated in this research, it is likely that additional waxy lines could be identified within the U.S. sorghum collection by application of reliable screening procedures. This investigation also demonstrates, however, that SDS-PAGE evaluation of starch granule proteins alone would not correctly identify all

waxy sorghum accessions. Several GBSS+, but amylose-free lines, were detected.

Before this research, the BTxARG-1 and B9307 (an experimental line from Texas A&M University) were known to be waxy yet still possess GBSS (Pedersen et al., 2005). Seed of both lines was found to contain evidently nonfunctional GBSS. The exact pedigree of B9307 is unknown, but is thought to be similar to BTxAGR-1 (William L. Rooney, personal communication, 2002). The discovery of GBSS in PI 217897 from Indonesia and PI 23231, PI 563670, and PI 563671 from China more than doubles the number of lines known to possess the wx^b allele. The presence of wx^b in lines from China and Indonesia would indicate either two independent mutations or germplasm flow across a fairly wide distance.

The observed gelatinization temperatures of both waxy classes were similar to those reported for waxy sorghum by Choi et al. (2004). The observed differences between the two waxy genotypic classes in both onset and end temperatures suggests a possible role of GBSS in maintenance or alteration of starch structure, as proposed by previous investigators (Hamaker and Griffin, 1993; Hamaker et al., 1991). Han and Hamaker (2002) further demonstrated a role of GBSS in maintaining the structure of starch "ghosts" or gelatinized starch gran-
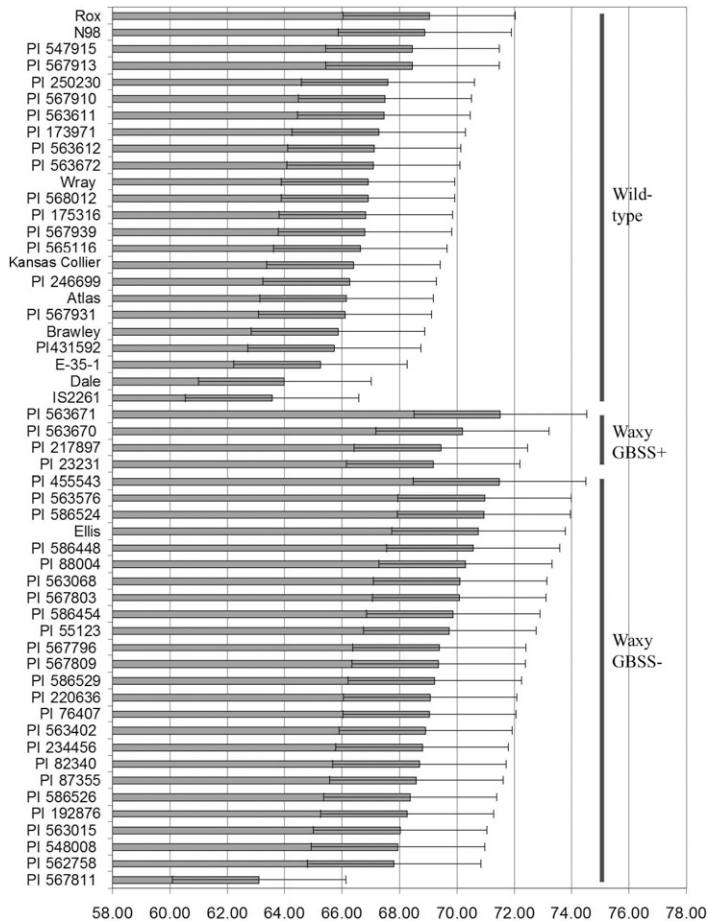
Figure 1. Least square means and standard errors for gelatinization onset temperature (°C). GBSS, granule bound starch synthase.
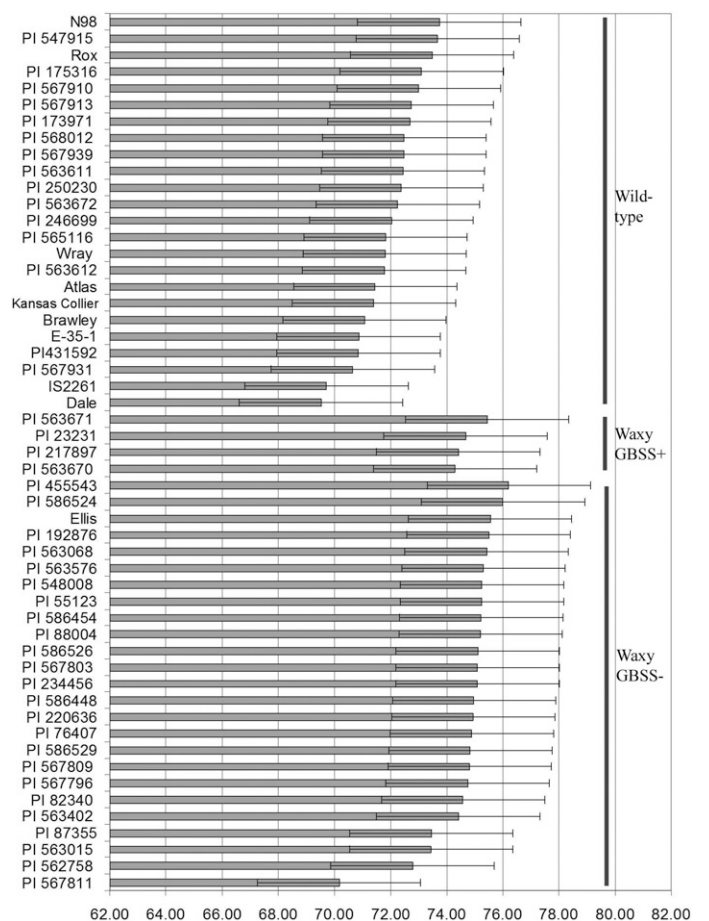
Figure 2. Least square means and standard errors for gelatinization peak temperature (°C). GBSS, granule bound starch synthase.
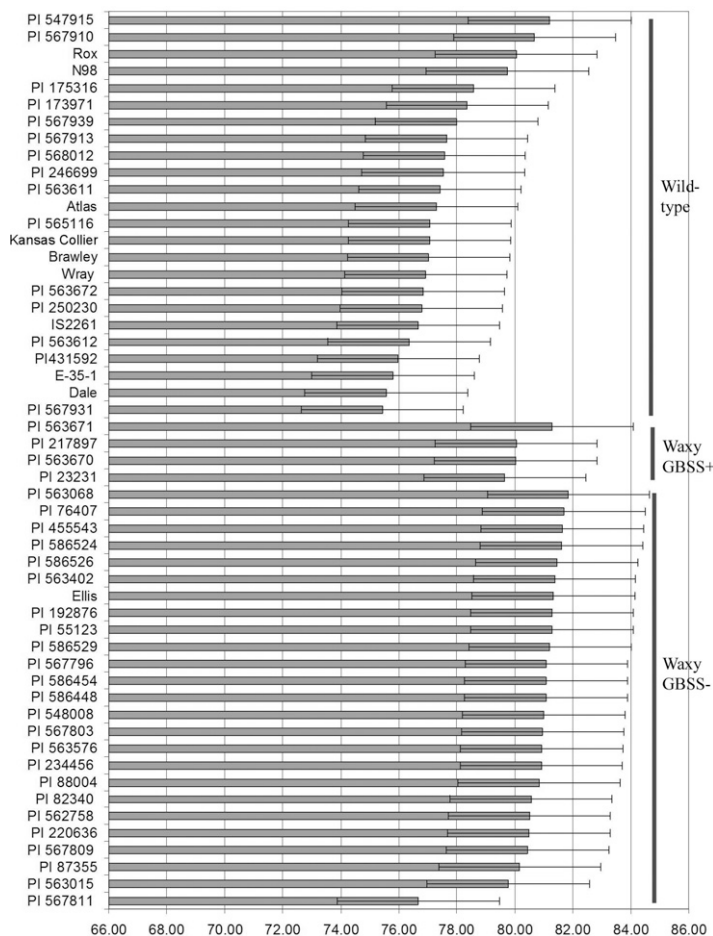
Figure 3. Least square means and standard errors for gelatinization end temperature (°C). GBSS, granule bound starch synthase.

Figure 4. Least square means and standard errors for gelatinization enthalpy (J g^{-1}). GBSS, granule bound starch synthase.

ule remnants. van de Wal et al. (1998) hypothesized that during the process of starch granule synthesis, GBSS becomes tightly and perhaps covalently attached to the amylopectin matrix. In sorghum lines carrying the wx^b allele, GBSS is present, but not functional. It also is not extracted with SDS solutions and is only liberated from the starch granule after boiling. Even though it evidently lacks enzymatic activity, GBSS in wx^b waxy sorghum may bind tightly enough to amylopectin to alter its melting parameters. There may be, therefore, microstructural differences between the waxy starches of different sources in sorghum, and these differences might result in different functional or digestive properties.

Significant genetic variation was observed within genotypic classes, especially for gelatinization enthalpy (Fig. 4), suggesting either influence of additional modifier genes affecting sorghum starch structure, or micro-environmental effects. Jennings et al. (2002) found harvest date to have a significant effect on DSC–determined enthalpies of maize starches, with later harvested materials requiring more energy for transition. Differences in days to maturity amongst this diverse sorghum germplasm collection also might have influenced starch granule melting properties. Standard errors for DCS
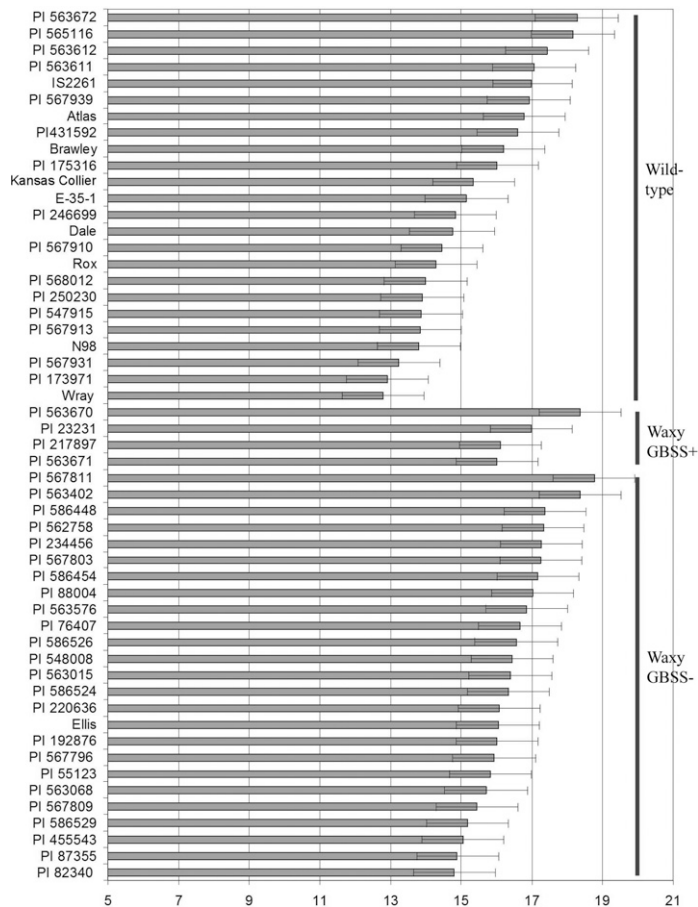
values were larger than generally reported. Environmental effects of two production years plus micro-environmental effects of field location would be expected result in larger standard errors than in studies that do not include these sources of variation.

In North America, the commercial starch industry primarily relies on maize, utilizing wild-type and various mutant forms including high-amylose and waxy. In agroecological terms, sorghum is an attractive alternative to maize. Sorghum may be produced in most environments used to cultivate maize, but sorghum requires far less water. Under periods of prolonged drought, sorghum provides producers an attractive alternative. The observed variation in starch properties reported herein, both within wild-type and waxy classes, suggests the availability of sorghum accessions useful in diverse starch products. Presently, sorghum primarily is used as a feed grain for livestock. However, worldwide, sorghum is used to produce unleavened breads, alcoholic beverages, or in the manufacture of building materials. Finally, the detected differences in starch gelatinization properties of GBSS+ and GBSS− waxy genotypes will aid in further elucidation of the role of granule-bound proteins in starch granule formation and structure.
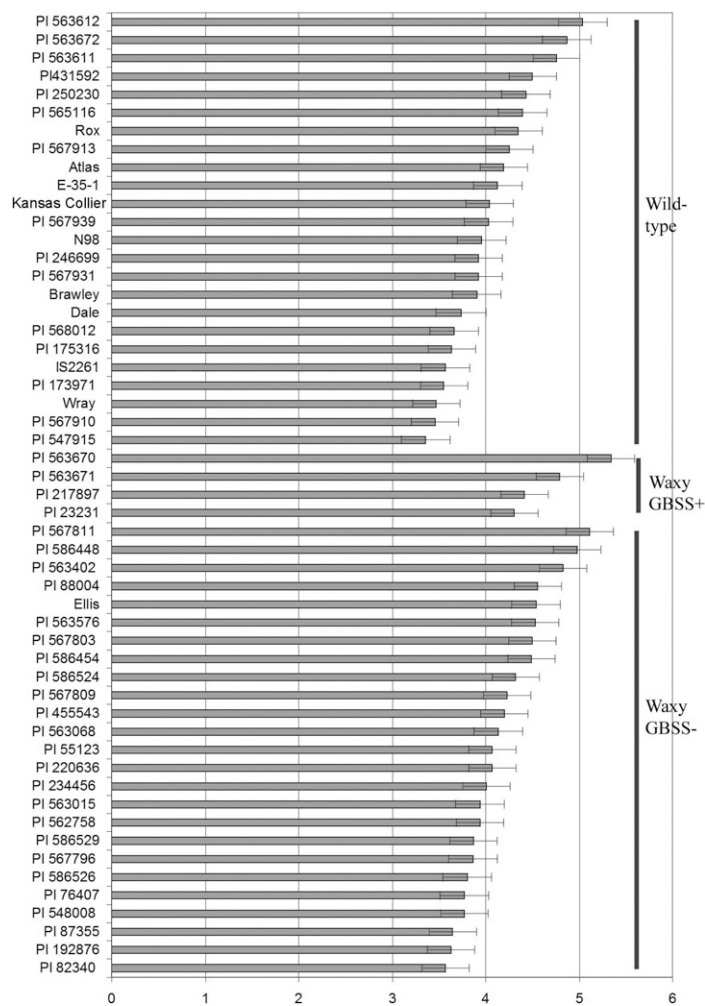
Figure 5. Least square means and standard errors for peak height (mW). GBSS, granule bound starch synthase.

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