Identification and Characterization of U.S. Wheats Carrying Null Alleles at the *wx* Loci[1]

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ABSTRACT

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Granule-bound starch synthase (GBSS) is the primary enzyme responsible for the synthesis of amylose in amyloplasts of cereal endosperm cells. Bread wheats, due to their hexaploid genetic system, carry three genes (*wx* loci) encoding GBSS. Purification and separation of GBSS from more than 200 North American hexaploid wheats allowed the identification of genotypes that carry null alleles at either the *wx-A1* and *wx-B1* loci. In addition, the cultivar Ike carried both *wx-A1* and *wx-B1* null alleles. No *wx-D1* nulls were detected. Null alleles were found in 10% of the hard winter wheats tested, but in only 2% of the sampled soft winter wheats. Amylose contents of wheats carrying single null alleles at either the *wx-A1* or *wx-B1* loci often were lower than those of wild type wheats, but greater reduction in amylose content was observed in Ike. Monoclonal antibodies were used to quantify water-extractable GBSS in both wild-type and null genotypes. Gene dosage compensation was evident, although GBSS content, as measured by ELISA, was significantly lower in Ike than in all other wheats. The identification of null alleles in adapted genotypes suggests the development of wheats with a wide range of amylose contents will be possible by simple genetic crossing and selection.

Starch consists essentially of two types of polymers of glucose, linear amylose and branched amylopectin. In maize and wheat (among other plants), it has been shown that the loss of granule-bound starch synthase (GBSS) from the endosperm results in the absence of amylose in the starch (Echt and Schwartz 1981, Nakamura et al 1995). Furthermore, in rice (Shimada et al 1993) and potato (Visser et al 1991), the introduction of an antisense gene to GBSS can result in the drastic reduction in the amount of amylose produced. In common wheat (*Triticum aestivum* L.), there are three loci (*wx-A1*, *wx-B1*, *wx-D1*) encoding GBSS (Yamamori et al 1994). Recent advances in the purification and electrophoretic separation of wheat GBSS now allow the identification of the gene products of the three *wx* loci (Nakamura et al 1992, 1993). In addition, Nakamura et al (1992, 1993) and Yamamori et al (1994) identified a number of wheat lines that carry null alleles at one of the three loci. Null alleles at *wx-A1* and *wx-B1* were found to be fairly common among wheats of Japanese and Australian origin, respectively. Only one line, Bai Huo, from China, was identified as possessing a null allele at the *wx-D1* locus. Lines carrying one or two null alleles were designated “partial-waxy” lines and often produced starches with reduced amylose (Nakamura et al 1992, 1993; Yamamori et al 1994). Hybridization of lines carrying null alleles has resulted in the production of amylose-free (waxy) common and durum wheats (Nakamura et al 1995).

The potential use of wheat starch with reduced amylose contents is a current topic of discussion among wheat breeders and geneticists. Reduced-amylose (partial-waxy) wheats have been shown to confer superior performance in Udon noodle applications (Miura and Tanii 1994) and may confer both extended shelf-life and enhanced water absorption to baked goods. Amylose-free starch might find applications in both paper and food industries, and most likely would be useful in all present applications of waxy maize starch. Rapid development of both reduced-amylose and amylose-free wheat cultivars for the United States would be facilitated by the identification of adapted lines carrying one or more *wx* null alleles. The goals of the present study were to: 1) survey recently released U.S. cultivars and advanced breeding lines to identify lines carrying *wx* null alleles, 2) characterize wheats with *wx* null alleles in terms of both amylose and GBSS contents, and 3) compare the relative effects of environment, genotype, and genotype × environment interaction on starch amylose and GBSS contents. Eventual goals are the development of a series of reduced-amylose and amylose-free hard and soft winter wheats through intermatings of wheats with the various GBSS null alleles.

MATERIALS AND METHODS

Wheat lines surveyed included 196 hard winter wheat cultivars and advanced experimental lines of Great Plains origin, and 50 soft winter wheats from the eastern or southeastern U.S. Grain samples (5 g) were ground in a Udy cyclone mill fitted with a 1.0-mm screen, starch was purified (Nakamura et al 1992), and GBSS was extracted and separated by SDS-PAGE according to Nakamura et al (1992) with the following modifications. GBSS disulfides were reduced with dithioerythritol and alkylated with 4-vinyl-pyridine by the procedure used by Graybosch and Morris (1990) for reduction and alkylation of wheat gluten proteins. Gel and run buffer formulations also were those of Graybosch and Morris (1990). GBSS nulls were identified by comparisons to the wild-type (three active genes), cultivar Scout 66, and to the double null line Kanto 107. Kanto 107 carries null alleles at both the *wx-A1* and *wx-B1* loci (Nakamura et al 1993).

Amylose contents of starches from wheats with various null alleles were measured in three sets of experimental materials. Experiment 1 included single samples of 13 experimental lines and cultivars grown in 1994 at Berthoud, CO. Samples were obtained from Rob Bruns of AgriPro (Berthoud, CO). Genotypes in Experiment 1 included wild-type *wx-A1* and *wx-B1* single nulls, and one double-null line (null at both *wx-A1* and *wx-B1*). Experiment 2 contained 11 wheat lines sown in two replicate trials at three Nebraska locations (Lincoln, Sidney, and Scottsbluff) in both 1990 and 1991; wild type and *wx-A1* and *wx-B1* single null lines were included. Experiment 3 consisted of 13 wheats sown in three replicate trials at three Kansas locations (Graham, Barton and Tribune counties) in 1995. Samples were obtained from Joe Martin, Kansas State University, Hays, KS. Experiment 3 contained wild-type, single, and double null wheats. Apparent amylose contents were determined by I_2 binding after dissolution of starch in dimethylsulfoxide (Knutson and Grove 1994). Amylose contents were expressed as percent of total starch.

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The relative amount of water-extractable GBSS protein in wheat lines with various numbers of active GBSS alleles also was determined. Two monoclonal antibodies (mabs) specific for GBSS (Rahman et al 1995) were used to develop a sandwich enzyme-linked immunosorbent assay (ELISA). Mab 91484, diluted to a concentration of 3.0 µg/mL in 1× PBS (130 m*M* NaCl, 1.8 m*M* KH_2PO_4, 10.1 m*M* Na_2HPO_4) was used as a capture antibody. GBSS was extracted by boiling 10 mg of starch for 10 min in 1 mL of H_2O, followed by centrifugation at 14,000 × *g* for 30 min. The resultant supernatant was diluted 1/10 in H_2O before application to mab 91484-coated Nunc (Denmark) C96 Maxisorp microwell plates. Mab 91563 (0.5 µg/mL in 1× PBS) labeled with horseradish peroxidase was used as the tag (reporter). Substrate for the enzyme was 2 m*M* diammonium 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma, St. Louis, MO), 3% (w/v) hydrogen peroxide, and 0.05*M* Na-citrate, pH 4.5. Reactions were stopped after 30 min by addition of 3% oxalic acid. Relative absorbencies at 405 nm were converted to micrograms of water-extractable GBSS per milligram of starch through use of standard curves developed with GBSS from starch of Scout 66. Based on densitometric scans, boiling starch in water extracts ≈35–40% as much GBSS as does boiling in either 1 or 10% SDS solutions (data not shown). Unfortunately, SDS also interfered with detection of GBSS by antibodies in the ELISA. The ELISA, therefore, measures only water-extractable GBSS. References in this article to "GBSS contents" refer only to this portion of total GBSS.

SAS programs and procedures (SAS Institute, Cary, NC) were used in statistical analyses. Analysis of variance (ANOVA) and calculation of least significant differences (LSD) were used to compare mean responses from both amylose determinations and ELISA. Experiment 1 was analyzed as a randomized complete block design with three laboratory replicates. Experiments 2 and 3 were treated as randomized complete blocks with environment, replicate within environment, and environment × line as random effects, and line as a fixed effect in the model. In Experiment 2, each location-year combination was treated as a separate environment. Appropriate error terms were used to test significance of main effects. In this article, line is used to designate a specific wheat cultivar or experimental line, and genotype is used to designate GBSS allelic composition.

RESULTS AND DISCUSSION

Null alleles (Fig. 1) at two of the three *wx* loci were detected among U.S. hard wheats (Table I). Of 196 hard winter wheats assayed, 21 (10.7%) carried a null allele at least in one of the *wx* loci; seven carried a *wx-A1* null, while 13 lines possessed a null allele at the *wx-B1* locus. In addition, the cultivar Ike carried null alleles at both *wx-A1* and *wx-B1*. Among the 50 soft winter wheats, only one line (LA8676-B-21-4-1-B, null *wx-A1)* carrying a null allele was detected. No soft wheats with *wx-B1* nulls were identified, and no *wx-D1* nulls were detected in either class of wheat.

Significant differences in both amylose and GBSS contents were observed among the lines entered in Experiment 1 (Table II). Ike, the cultivar carrying null alleles at both *wx-A1* and *wx-B1*, displayed amylose and GBSS contents both significantly lower than all other lines (Table II). With the exception of Laredo, mean amylose contents of all single null lines were lower than that of Hawk and Jagger, the wild-type lines with the highest amylose contents. However, no significant differences were observed between the single null lines and the remaining wild-type lines. Among single null and wild-type lines, a continuous range in GBSS content, as measured by ELISA, was observed, and few significant differences were detected.

In Experiment 2, ANOVA (Table III) demonstrated significant effects of environment, line, and line × environment interaction on amylose content. Hence, genetic factors (i.e., allelic status at the *wx* loci) do not explain all the variation in amylose content. In contrast, environment and line × environment interactions did not significantly affect GBSS contents. Significant differences, however, were observed among lines (Table III). Mean amylose contents of

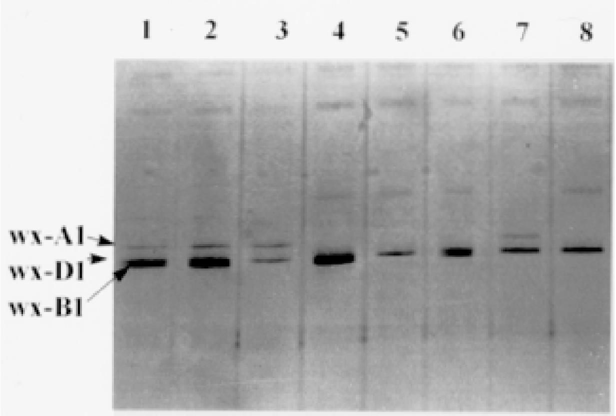

Fig. 1. SDS-PAGE separation of granule-bound starch synthase from various wheat cultivars. Gene products of the three wheat *wx* loci are indicated.

TABLE I
U.S. Hard Winter Wheats Carrying *wx* Null Alleles

Genotype	Lines
wx-A1 null	Chisholm, Cimarron, KS801072 Colt, NE86501, Laredo, Custer
wx-B1 null	TAM200, TAM202, TX92V3108, TX93V5919, TX93V5922, TX93V4927, RioBlanco, WI93335, WI93339, K94H115, K94H400, K94H402, CO910748
wx-A1 null, *wx-B1* null	Ike

TABLE II
Amylose and Granule-Bound Starch Synthase (GBSS) Contents from 13 Wheat Lines Obtained from Berthoud, CO

Line	Genotype	Amylose Content (%)	GBSS Content (µg/mg of starch)
Hawk	Wild-type	34.04	0.83
WI89163W	Wild-type	30.95	0.72
WI90540	Wild-type	31.08	0.79
WI91103	Wild-type	29.71	0.92
WI93357	Wild-type	30.28	0.87
WI93481	Wild-type	32.44	0.97
Jagger	Wild-type	33.72	0.92
Laredo	Null *wx-A1*	29.10	0.94
Custer	Null *wx-A1*	28.12	0.91
RioBlanco	Null *wx-B1*	28.70	0.71
WI93335	Null *wx-B1*	28.48	0.75
WI93339	Null *wxB1*	26.41	0.84
Ike	Null *wx-A1*, Null *wx-B1*	19.74	0.59
Least significant difference		5.16	0.12

TABLE III
Mean Squares from Analysis of Variance of Amylose and Granule-Bound Starch Synthase (GBSS) Contents of 11 Wheat Lines Grown at Three Nebraska Locations, in 1990 and 1991

		Mean Squares	
Source of Variation	df	Amylose	GBSS
Environment	5	116.20*[a]	1.54
Replicate (environment)	6	15.53	0.64
Line	10	45.10*	1.17*
Line × environment	50	15.31*	0.60

[a] * = Significant at *P* = 0.05.

two of the single null lines, TAM200 and KS801072, were significantly lower than those of all wild-type lines with the exception of TAM107 (Table IV). A similar result was obtained with the ELISA, except GBSS content of KS801072 did not differ from that of most wild-type lines.

In Experiment 3, ANOVA did not detect significant effects of environment or line × environment interaction for either amylose or GBSS content (Table V). In Experiment 3, however, lines were harvested from three locations within only one harvest year; in contrast, Experiment 2 contained lines from three locations but with two harvest years represented. The larger number of sampled environments (year × location combination) could explain the differential magnitude of the environmental effects in these two experiments. Significant differences in amylose and GBSS contents were observed among lines in Experiment 3 (Table VI). Again, Ike had the lowest amylose content; KS94HW115, a *wx-B1* null, did not differ from Ike in amylose content. In the ELISA, Ike displayed significantly lower GBSS than all other lines. The two *wx-B1* lines included, KS94HW115 and RioBlanco, had greater amounts of GBSS than Ike, but lower than amounts observed in all wild-type wheats.

Across the three experiments, the 19.74–34.04% range of amylose contents observed was slightly greater than that reported by Yamamori et al (1994) for Japanese cultivars. In general, amylose contents of wheat >30% are rare (Lineback and Rasper 1988), although in the current study, several were observed. More importantly, the distribution of amylose contents among wheats with various GBSS allelic compositions was nearly identical to that reported by Yamamori et al (1994) for Japanese wheats. Yamamori et al (1994) also observed that the lowest amylose contents occur in double null lines, with some single null lines being lower than wild-type, but a zone of overlap of wild-type and single nulls was evident in both the present study and that of Yamamori et al (1994).

Approximately 10% of the sampled hard winter wheats carried null alleles at either the *wx-A1* and *wx-B1* locus. Results of the three experiments suggest the designation of lines carrying single null alleles as partial waxy (Nakamura et al 1992, 1993) might be inappropriate. While some of the lines carrying single null alleles displayed significantly lower amylose contents than the majority of wild-type wheats, the effect was not universal and the amylose depression was slight. In contrast, starch of Ike contained ≈20% amylose, slightly more than two-thirds that of the majority of wild-type wheats. The partial waxy designation might be more appropriately applied only to double null lines. While genetic variation did exist among single null lines for amylose content, and lines with significantly lower amylose than most wild-type lines were identified, the significant effects of environment and line × environment interactions in Experiment 2 suggest single null lines might be unreliable as sources of low amylose wheat starch. In this regard, production of adapted double null lines, through intermatings of single null wheats will be the more likely consistent source of low amylose starch.

The ELISA data suggest that active *wx* alleles are capable of dosage compensation; the amount of GBSS was not entirely a function of the number of active genes. In some lines with null alleles, GBSS content was equal to that of wild-type. The presence of a single null allele did not always reduce GBSS production to an extent that a concomitant reduction in amylose content was detected. GBSS content of Ike was ≈50% that of wild-type lines. These conclusions assume a direct relationship between the amount of water-extractable GBSS and total GBSS. Such a relationship needs to be confirmed with future experimentation. Regardless of the results of such studies, the sandwich ELISA could be useful as an alternative to SDS-PAGE as a means of identifying double null lines if subsequent studies with additional double null lines confirm the observations made with Ike.

The single and double null cultivars and advanced breeding lines identified herein will be extremely useful in the development of locally adapted wheats carrying reduced-amylose or amylose-free starch. Double null lines likely will be the best source of reduced-amylose starch. However, additional studies, designed to compare the effects of the various null alleles in segregating genetic populations, and among more genetic backgrounds, are necessary. Both single and double null lines also will be useful as parents in the development of amylose-free cultivars. Nakamura et al (1995) have produced amylose-free common wheat through intermating of Kanto107, a Japanese wheat carrying both *wx-A1* and *wx-B1* null alleles, and Bai Huo a wheat of Chinese origin carrying a *wx-D1* null. While this mating is useful as the intitial source of amylose-free wheat starch, commercial availability will depend upon the introgression of the null alleles to locally adapted cultivars. The adapted lines with null alleles identified in this article will greatly decrease the time frame necessary for this task.

TABLE IV
Mean Amylose and Granule-Bound Starch Synthase (GBSS) Contents of 11 Wheat Lines Grown at Three Nebraska Locations, in 1990 and 1991

Line	Genotype	Amylose Content (%)	GBSS Content (µg/mg of starch)
Centurk 78	Wild-type	30.37	1.21
Redland	Wild-type	30.21	1.06
Scout 66	Wild-type	29.61	1.07
Siouxland	Wild-type	30.30	1.01
TAM107	Wild-type	27.49	1.01
Vona	Wild-type	29.79	1.05
Chisholm	Null *wx-A1*	28.56	1.02
Cimarron	Null *wx-A1*	29.25	1.06
KS801072	Null *wx-A1*	25.17	1.01
TAM200	Null *wx-B1*	25.41	0.95
TAM202	Null *wx-B1*	26.81	0.95
Least significant difference		3.21	0.10

TABLE V
Mean Squares from Analysis of Variance of Amylose and Granule-Bound Starch Synthase (GBSS) Contents of 12 Wheat Lines Grown at Three Nebraska Locations, in 1995

		Mean Squares	
Source of Variation	df	Amylose	GBSS
Environment	2	77.08	0.03
Replicate (environment)	6	80.52	0.01
Line	11	61.42*[a]	0.21*
Line × environment	22	29.26	0.01

[a] * = Significant at $P = 0.05$.

TABLE VI
Mean Amylose and Granule-Bound Starch Synthase (GBSS) Contents of 12 Wheat Lines Grown at Three Nebraska Locations, in 1995

Line	Genotype	Amylose Content (%)	GBSS Content (µg/mg of starch)
Arlin	Wild-type	30.56	0.71
KS94HW123	Wild-type	29.77	0.73
KS94HW127	Wild-type	29.47	0.71
Jagger	Wild-type	28.71	0.71
KS94HW131	Wild-type	28.35	0.72
KS94HW119	Wild-type	28.02	0.69
RioBlanco	Null *wx-B1*	27.08	0.57
KS94HW66	Wild-type	26.50	0.75
KS94HW100	Wild-type	26.45	0.72
KS94HW101	Wild-type	26.05	0.74
KS94HW115	Null *wx-B1*	24.80	0.51
Ike	Null *wx-A1*, Null *wx-B1*	20.93	0.40
Least significant difference		4.14	0.06

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