Variability in Arabinoxylan, Xylanase Activity, and Xylanase Inhibitor Levels in Hard Spring Wheat

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ABSTRACT

Arabinoxylans (AX), xylanase, and xylanase inhibitors have an important role in many cereal food processing applications. The effects of genotype, growing location, and their interaction (G × L) on AX, apparent xylanase activity, and apparent xylanase inhibition activity of *Triticum aestivum* xylanase inhibitor (TAXI) and xylanase inhibiting protein (XIP) were investigated for six hard red and six hard white spring wheat genotypes grown at three locations. Difference in total AX level among genotypes was not determined to a significant level by genotype. Instead, variability in total AX content was largely dependent on G × L. However, total AX content was significantly different between the two wheat classes. For bran xylanase activity, 25% of the variability could be attributed to G × L interaction. Moreover, there was significant difference between the bran xylanase activities in the two wheat classes. Bran TAXI activity and XIP activity were significantly different among genotypes. Genotype contributed 72% to the variability in TAXI activity and 39% in XIP. However, no significant difference was observed among the two wheat classes for TAXI or XIP activity. These results indicate that TAXI might be a stable parameter in segregating wheat genotypes with varying xylanase activity.

Arabinoxylans (AX), xylanase, and xylanase inhibitors have an important role in many cereal food processing applications, including breadmaking (Courtin and Delcour 2002), gluten–starch separation (Frederix et al 2004), refrigerated dough (Courtin et al 2005; Poulsen and Sorensen 2006; Simsek and Ohm 2009), malting and brewing (Dornez et al 2009), and feed conversion of animal feeds (Bedford and Schulze 1998). Microbial xylanases are routinely added in some food processes to hydrolyze AX. However, the effectiveness of these additions is influenced by grain-associated xylanase and xylanase inhibitors occurring naturally in wheat kernels in variable amounts in different cultivars (Bonnin et al 2005; Gebruers et al 2010).

One of the major problems in wheat milling and processing industries is the variability in end-use quality of the wheat (Dornez et al 2008). Minor constituents such as lipids, enzymes, and nonstarch polysaccharides contribute to this variation (Dornez et al 2008). Of the nonstarch polysaccharides, AX are of great importance, as they are the most abundant component in the cell wall of wheat (Goesaert et al 2005). Even though they are a minor constituent of the grain, their water-holding capacity and viscous properties impose considerable impact on the functional properties of grain-based product processing. Furthermore, consumption of AX has been associated with beneficial health effects in humans (Garcia et al 2006). Xylanases, *endo*-β-(1,4)-D-xylanase (EC 3.2.1.8), are the main enzymes involved in hydrolysis of AX. They cleave AX by internally hydrolyzing the 1,4-β-D-xylosidic linkage between xylose residues in the xylan backbone (Collins et al 2005; Dornez et al 2009). This cleaving causes drastic changes to AX molecular weight, water extractability, and functional properties (Courtin and Delcour 2002). Thus, grain-associated xylanases in wheat kernels are capable of imparting significant effects on processing.

Apart from AX and xylanase, wheat kernels also contain high levels of xylanase inhibitors. These inhibitors inactivate grain-associated xylanases of microbial origin and hence affect the net functionality of xylanase in wheat processing (Dornez et al 2010). In wheat, three proteinaceous xylanase inhibitors have been identified to date: TAXI (*Triticum aestivum* xylanase inhibitor) (Debyser et al 1999), XIP (xylanase inhibiting protein) (McLauchlan et al 1999), and TLXI (thaumatin-like xylanase inhibitor) (Fierens et al 2007). The total content of these inhibitors is high in wheat (Dornez et al 2009), and considerable differences exist in the levels present in different cereal types and varieties (Gebruers et al 2010). Xylanase inhibitors in plants have been suggested to play a role of plant defense to disease (Dornez et al 2010). This theory is mainly supported by the fact that TAXI, XIP, and TLXI do not inhibit endogenous wheat xylanase but inhibit microbial xylanases. Some microbial xylanases are routinely added to wheat flour or whole meal to achieve the desired level of AX degradation. However, their effectiveness is in many instances affected by the level of inhibitors naturally occurring within the grain and the sensitivity of the microbial xylanases to these inhibitors. The increase in bread loaf volume upon addition of xylanase can be cancelled by adding TAXI (Debyser et al 1999). Xylanase inhibitors have been found to negatively affect the activity of added xylanase in pig and poultry feeds (Sørensen et al 2004). However, xylanase inhibitors have positive effects in some food processes. In refrigerated dough, addition of TAXI-type inhibitors has been shown to reduce syruping up to 50% after 10 days of storage at 10°C (Poulsen and Sorensen 2006).

XIP inhibits both glycoside hydrolase (GH) family 10 and GH family 11 xylanases, but only those of fungal origin (Dornez et al 2009), whereas TAXI-type inhibitors inhibit xylanases of fungal and bacterial origin from GH family 11 and not GH family 10 (Goesaert et al 2001). This understanding about the inhibition specificity of the xylanase inhibitors has been exploited to determine the levels of each inhibitor when both are present. Based on this specificity, Dornez et al (2006b) used *Bacillus subtilis* xylanase (GH 11) to determine TAXI activity and *Penicillium purpurogenum* xylanase (GH 10) to determine XIP activity in wheat grains.

Although AX, xylanase, and xylanase inhibitors play an important role in wheat processing, little research has been done on the contribution of genetic and environmental factors to their variability in hard spring wheat. Studies in these areas are important

*The *e*-**X**tra logo stands for "electronic extra" and indicates that Figures 2, 5, and 6 appear in color online.

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because factors that are under genetic regulation can be manipulated by wheat breeders to develop desired varieties. Moreover, knowledge on the contribution of genotype and environment interaction is also important in deciding the potential of newly developed varieties by breeders. With this possibility in mind, the main objective of the research was to determine the influence of genotype, growing location, and growing location × genotype interaction (G × L) on the variability of total AX, xylanase activity, and xylanase inhibition activity in hard red spring wheat (HRSW) and hard white spring wheat (HWSW) samples grown in North Dakota. The effect of location and genotype on the substitution pattern of arabinose in AX was also evaluated. To our knowledge, no research has been done to compare xylanase levels in HRSW and HWSW genotypes grown in North Dakota. Also, no data are available regarding the TAXI and XIP inhibition activity levels in North American–grown HRSW and HWSW genotypes.

MATERIALS AND METHODS

Materials

All the chemicals and reagents were of analytical grade. The spring wheat-breeding program at the Department of Plant Science, North Dakota State University, kindly provided all the wheat samples. Twelve genotypes were grown at three locations (Casselton, Carrington, and Prosper, ND) utilizing a randomized complete block design with four replicates in 2008. Wheat samples were six HRSW (Alsen, Briggs, Glenn, Granite, Hanna, and Ingot) and six HWSW (AC Snowbird, Explorer, Lolo, CS3100Q, Pristine, and 99S0155-14W) genotypes that were adapted to the U.S. Spring Wheat region. Out of the four replicates, replicates 1 and 2 were combined into one, and replicates 3 and 4 were combined into one to obtain two replicates.

The GH 10 *P. purpurogenum* xylanase (NCBI accession AAF-71268) was a kind gift from J. Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile). GH 11 *B. subtilis* xylanase (NCBI accession AAA22897) was from Danisco (Brabrand, Denmark).

A xylanase (Xylazyme AX) assay kit for determining *endo*-1,4-β-D-xylanase activity was purchased from Megazyme (K-XYLS, Megazyme, Bray, Ireland).

Weather Conditions During Cereal Growth

Temperature and rainfall data in the 2008 growing season were recorded (Rugg 2011). Each location received lower than average precipitation in the early part of the growing season. Temperatures were below normal at both environments in the early part of the growing season. Prosper had a higher precipitation and a higher temperature than Carrington, which may have resulted in higher disease pressure in Prosper than in Carrington. Because of the proximity of the location, data from Prosper was used for Casselton, too.

Sample Preparation

HRSW and HWSW samples were cleaned by passing through a dockage tester (XT5 3/03, Carter Day International, Minneapolis, MN, U.S.A.). Cleaned samples (150 g) were collected in glass jars and conditioned to 16.0% moisture content the day before milling. The samples were milled in a Quadramat Jr. laboratory mill (C.W. Brabender Instrument, South Hackensack, NJ, U.S.A.) to produce flour and bran. Bran was again ground in a cyclone sample mill with a 0.5 mm screen (UDY, Fort Collins, CO, U.S.A.) to reduce particle size. Another 50 g each of cleaned wheat samples were ground in a cyclone sample mill with a 0.5 mm screen (UDY) to produce whole meal. All samples were stored in sealed plastic bags at 4°C until analysis. Moisture content of all the samples was determined following AACC International Approved Method 44-15.02.

Determination of Total AX and Arabinose-to-Xylose Ratio of Wheat Bran with Gas Chromatography

Total AX (TOT-AX) and the ratio of arabinose to xylose (A/X) in AX in wheat bran were determined following acid hydrolysis and preparation of alditol acetates as described by Blakeney et al (1983). Arabinose and xylose were used as monosaccharide standards in the analysis. *Myo*-inositol was used as the internal standard. The derivatized alditol acetate samples were analyzed on a Hewlett Packard 5890 series II gas chromatography (GC) system with a flame ionization detector (Agilent Technologies, Santa Clara, CA, U.S.A.). A Supelco SP-2380 fused silica capillary column (30 m × 0.25 mm × 2 μm) (Supelco Bellefonte, PA, U.S.A.) was used in the GC system. The system parameters were as follows: flow rate, 0.8 mL/min; flow pressure, 82,737 Pa; oven temperature, 100°C; detector temperature, 250°C; and injector temperature, 230°C. The carrier gas was helium. AX was calculated according to the following formula (Henry 1986): TOT-AX = (% arabinose + % xylose) × 0.88.

Determination of Arabinose Substitution Pattern with Proton Nuclear Magnetic Resonance Spectroscopy

The arabinose substitution pattern of water-extractable AX (WE-AX) of wheat bran was determined through proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy following the method of Cleemput et al (1995). WE-AX was extracted from wheat bran samples. As bran was obtained through milling, bran WE-AX also contained some endosperm WE-AX. Samples of WE-AX (5 mg) extracted from wheat bran were dissolved in 700 μL of deuterium oxide (D_2O) with continuous stirring at 40°C for 60 min and freeze-dried. The freeze-dried samples were again dissolved in 700 μL of D_2O with continuous stirring at 40°C for 60 min and freeze-dried. The freeze-dried samples were dissolved in 800 μL of D_2O with continuous stirring at 40°C for 60 min and transferred to NMR tubes. ^{1}H-NMR spectra were obtained with a Varian Unity Inova 500 MHz NMR spectrometer (Varian, Palo Alto, CA, U.S.A.) at 80°C.

Determination of Apparent Xylanase Activity

Sample Preparation. Whole meal (2.0 g), flour (2.0 g), and bran (1.0 g) were extracted for 60 min at 6°C in 20 mL of sodium acetate buffer (25m*M*, pH 4.7, 0.02% NaN_3) on a horizontal shaker (150 rpm). The contents were centrifuged (16,000 × *g*, 4°C, 15 min) and the supernatants filtered through VWR Qualitative 413 filter paper (particle retention, 5–13 μm) to obtain the aqueous extracts that were used in the xylanase activity assay.

Apparent Xylanase Activity Assay. The Megazyme Xylazyme AX method was used with modifications to determine the apparent xylanase activity of whole meal, flour, and bran extracts as described by Gebruers et al (2010). An enzyme standard series (0.295, 0.059, 0.0295, 0.01475, and 0.007375 mU/mL) in sodium acetate buffer (25m*M*, pH 4.7, 0.02% NaN_3) was prepared using *Aspergillus niger* xylanase (295 mU/mL) provided with the Xylazyme AX assay kit (enzyme concentration in mU as specified by Megazyme). Standards were stored in the refrigerator at 4°C and were not used beyond two days from the date of preparation. One milliliter of the test sample (whole meal, flour, or bran extracts, buffer [blank], or the standard xylanase [*A. niger* xylanase] solution) was drawn into a test tube and equilibrated at 40°C for 10 min. One Xylazyme AX substrate tablet was added to each tube. After the addition of the tablets, the tubes were further incubated for 17 hr at 40°C. The reaction was stopped by adding 6.00 mL of Trizma base solution (165m*M*, pH 9.36). After 10 min at room temperature, the solutions were filtered through VWR Qualitative 413 filter paper (particle retention, 5–13 μm). The resultant solution was of pH 9.31. The absorbance was measured at 590 nm with a Hach DR/4000U spectrophotometer (Hach, Loveland, CO, U.S.A.) against a blank prepared with the aforementioned sodium acetate buffer. Corrections were made for nonenzymic color re-

lease by the tablets. Apparent xylanase activity of whole meal, flour, and bran was calculated from the standard series of *A. niger* xylanase solutions. Corrections were made for the moisture content of the initial samples, and apparent xylanase activity was expressed as milliunits per gram of dry matter (mU/g of d.m.) of sample.

Determination of Apparent Xylanase Inhibition Activity of TAXI and XIP

Extracts from flour (2.00 g) and bran (1.00 g) were obtained as described previously and used in the xylanase inhibition activity assays. TAXI and XIP activities were measured with the Xylazyme AX assay procedure described by Gebruers et al (2010). Xylanase with specific activity was used in the measurements. *B. subtilis* xylanase, which belongs to GH family 11, was used to measure TAXI activity, as this xylanase is inhibited only by TAXI. *P. purpurogenum* xylanase, which belongs to GH family 10, was used to measure XIP activity, as only XIP inhibits this xylanase (Gebruers et al 2010). *B. subtilis* xylanase and *P. purpurogenum* xylanase enzyme solutions were prepared in sodium acetate buffer (25m*M*, pH 4.7, 0.02% NaN_3) containing bovine serum albumin (0.500 g/L). The enzyme concentrations used resulted in an absorbance of 0.46 (*B. subtilis* xylanase) and 0.48 (*P. purpurogenum* xylanase) when used in the previously described assay of determination of apparent xylanase activity (corrections were made for nonenzymic color release). Xylanase solutions (0.5 mL) were preincubated for 30 min at 30°C with an equal volume of buffer to prepare the uninhibited reference. The control for the uninhibited reference was prepared with 0.5 mL of buffer instead of xylanase solution. Xylanase enzyme solutions (0.5 mL) were preincubated for 30 min at 30°C with an equal volume of diluted flour or bran extract to prepare the inhibited sample. The control for the inhibited sample was prepared with 0.5 mL of buffer instead of xylanase solution. Preincubation allowed time for formation of enzyme-inhibitor complex in the inhibited sample. A Xylazyme AX tablet was added to each tube following preincubation. Then the tubes were incubated for 60 min at 30°C. Then the reaction was stopped by adding 6.00 mL of Trizma base (165m*M*, pH 9.36). After 10 min at room temperature, the contents were filtered (VWR Qualitative 413 filter paper, particle retention, 5–13 μm). The absorbance of the samples and the reference at 590 nm was measured as described previously. Absorbance of the uninhibited reference was measured against the control for the uninhibited reference. Absorbance of each inhibited sample was measured against the corresponding control for the inhibited sample. Xylanase inhibition activity was expressed in inhibition units (InU) as defined by Gebruers et al (2010), where 1 InU is the amount of xylanase inhibitor needed to inhibit xylanase activity by 50% in the previously described assay.

Statistical Analysis

Statistical analysis was done with SAS (version 6.1 for Windows, SAS, Cary, NC, U.S.A.). Bartlett's test was performed to find the homogeneity of error variance among locations. When the error values were not homogeneous, natural log transformation of data was performed or an analysis of variance was performed for individual locations. The MIXED procedure was used for analysis of variance assuming that effects of genotype and location were random. The variance component values estimated from the MIXED procedure were used to calculate the percentages of contribution of genotype and location and their interaction (G × L) effects to the total variance.

RESULTS AND DISCUSSION

Total AX Contents and Arabinose-to-Xylose Ratio of Wheat Bran AX

AX play an important role in wheat-based products through their high water-absorbing capacity and their ability to form hydrated networks via chain entanglement or covalent bonds via ferulic acid residues in neighboring chains (Izydorczyk and Biliaderis 1992). Thus, evaluating the factors that can affect the structure of AX is important. Table I lists the TOT-AX (% of d.m.) and A/X ratio of AX of bran from 12 hard spring wheat genotypes harvested in 2008. TOT-AX in bran varied from 14.99% in Lolo to 27.69% in Explorer among genotypes. These values were somewhat higher than those reported by Gebruers et al (2010), whose values ranged from 12.1 to 19.2% of TOT-AX in bran for 20 spring wheat genotypes grown in Europe.

TOT-AX showed no significant difference (at $P < 0.05$) between genotypes (Table I) despite seemingly large differences. The effect of genotype was considered random in the analysis of variance, assuming that variation in AX levels of wheat genotypes employed in this research could be representative of HRSW and HWSW grown in North Dakota. Because the effect of genotype was random, the G × L interaction term was an error term to test significance of variance for genotypes in the MIXED procedure. Variance of TOT-AX could not be significant for genotypes because of the large variance in G × L interaction that contributed to approximately 66% of total variance in this research. Dornez et al (2008) analyzed six wheat varieties grown in Europe and found

TABLE I
Range and Mean Values of Total Arabinoxylans (TOT-AX) and Arabinose to Xylose (A/X) Ratio of Bran for 12 Hard Spring Wheat Genotypes[a]

	TOT-AX (% of d.m.)		A/X Ratio		Flour Yield (%)		Bran Ash (%) (12% mb)	
Genotype	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Alsen[b]	16.62–21.99	18.97	0.86–0.95	0.91	54.6–59.3	56.4	2.65–2.79	2.71
Briggs[b]	18.55–24.06	21.65	0.85–0.97	0.89	56.1–58.3	57.3	2.75–2.84	2.79
Glenn[b]	14.59–22.64	18.87	0.95–0.99	0.98	54.9–55.9	55.4	2.56–2.65	2.59
Granite[b]	13.68–20.53	18.21	0.90–0.98	0.95	52.5–54.6	53.3	2.78–2.87	2.81
Hanna[b]	16.04–24.45	20.92	0.82–0.99	0.91	58.0–60.9	59.7	2.75–2.76	2.76
Ingot[b]	22.45–23.97	23.24	0.84–0.93	0.89	56.3–61.7	58.6	2.63–2.69	2.67
AC Snowbird[c]	17.00–25.39	20.14	0.84–0.94	0.91	54.6–57.8	56.2	2.50–2.61	2.55
Explorer[c]	18.48–41.44	27.69	0.88–0.98	0.93	48.7–50.0	49.5	2.64–2.82	2.74
Lolo[c]	12.55–16.94	14.99	0.83–0.99	0.92	51.9–55.1	53.4	2.61–2.71	2.65
CS3100Q[c]	14.90–24.81	20.29	0.83–1.00	0.93	50.4–57.3	54.9	2.49–2.52	2.50
Pristine[c]	18.57–24.00	21.25	0.88–0.99	0.95	54.2–54.9	54.5	2.75–2.87	2.82
99S0155-14W[c]	22.55–27.70	25.58	0.80–0.84	0.82	51.3–56.9	53.5	2.90–3.03	2.94
LSD	…	ns	…	0.05	…	2.1	…	0.10

[a] Grown at three different locations (Carrington, Casselton, and Prosper, North Dakota) in 2008. LSD = least significant difference ($P < 0.05$); and ns = nonsignificant ($P > 0.05$).
[b] Hard red spring wheat.
[c] Hard white spring wheat.

that only 18% of the total variance in AX in their wheat samples could be explained by the genotype. Earlier, Andersson et al (1993) reported that no relation could be found between the non-starch polysaccharide levels and genotype in wheat grain. The combined analysis of variance indicated that the TOT-AX in the samples tested largely depended on the G × L effect. Even though there was no significant difference between genotypes, the TOT-AX content was significantly different between mean values of HRSW and HWSW wheat classes ($P < 0.01$).

The substitution pattern of AX influences the shape, solubility, and molecular characteristics of the AX polymer (Storsley et al 2003). In general, the presence of substitutions in the polymers limits their chain–chain interactions, which makes the polymer more water soluble. However, in the case of AX, water solubility is not only governed by the presence of substitution but also by the covalent linkages to other cell-wall components and other chains (Saulnier et al 2007). For example, whereas some WE-AX have a lower A/X ratio than some water-unextractable AX (WU-AX), high levels of diferulic covalent linkages among AX chains in WU-AX can render them water unextractable. A/X ratios of all the genotypes studied indicated a high level of arabinose substitution in bran AX. We also observed significant differences in A/X ratio between genotypes ($P < 0.05$) (Table I). This observation indicated that variation in substitution pattern in AX depended on genotype. Other researchers also found significant variation in AX substitution among varieties (Storsley et al 2003; Simsek and Ohm 2009). However, no such significant difference was observed for A/X ratio in bran AX between the wheat classes HRSW and HWSW.

Substitution Pattern of AX Analyzed by ^{1}H-NMR

WE-AX was extracted from bran samples belonging to four genotypes from all three locations and was analyzed with ^{1}H-NMR spectroscopy. Because of the milling process used, bran was contaminated with starchy endosperm. Therefore, bran WE-AX also contained endosperm WE-AX. Figure 1 shows a representative ^{1}H-NMR spectrum with resonance assignments. The spectrum consisted of two distinct groups of signals. The anomeric protons of α-L-arabinofuranosyl accounted for the resonances occurring at the range of δ5.2–5.4 ppm (Hoffmann et al 1992). The signals at the region δ4.4–4.7 ppm were attributed to the anomeric protons of β-D-xylopyranosyl residues. The anomeric proton of arabinose residue linked to the monosubstituted xylose at the O-3 position corresponded to the resonance at δ5.4 ppm (signal 1). The signal at δ5.3 ppm corresponded to the anomeric proton of arabinose residue linked to the disubstituted xylose at the O-3 position (signal 2), and the δ5.22 ppm peak was for the anomeric proton of arabinose residue linked to the disubstituted xylose at the O-2 position (signal 4). The anomeric proton of arabinose residue linked to the disubstituted xylose at the O-2 position, because the xylose had an adjoining disubstituted xylose residue, gave rise to the resonance (signal 3) between signals 2 and 4 at δ5.26 ppm. The anomeric proton of disubstituted, monosubstituted, and nonsubstituted xylose residues corresponded to signals 5, 6, and 7 respectively. The peak proportions of signals 1–4 in the ^{1}H-NMR were calculated. Of the two HRSW genotypes, Ingot had a tendency of having high proportions of disubstituted xylose with neighboring disubstituted xylose (signal 3) in all three locations. Also, the proportion of monosubstituted xylose in Ingot AX was relatively low. A similar trend was observed for the HWSW genotype Lolo. This observation indicated that AX in Ingot and Lolo were highly substituted. Differences in the proportion of substitution were seen between genotypes, but the variation was not constant across all three locations. It has been suggested that the distribution of arabinose along the xylose backbone is irregular (Dervilly-Pinel et al 2004). Random distribution of clusters with high substitution patterns and low substitution patterns has been identified in wheat AX (Gruppen et al 1993; Dervilly-Pinel et al 2004). This distribution might help explain the inconsistent variation in each substitution proportion seen across genotypes and across locations. However, the distribution of the type of substitution (mono- or disubstitution) is considered a nonrandom process (Gruppen et al 1993; Dervilly-Pinel et al 2004). The biosynthetic mechanism for AX indicates a preference for disubstitution (Dervilly-Pinel et al 2004). This trend was observed in our data too. The monosubstituted xylose proportion was the smallest proportion in all genotypes at all locations tested.

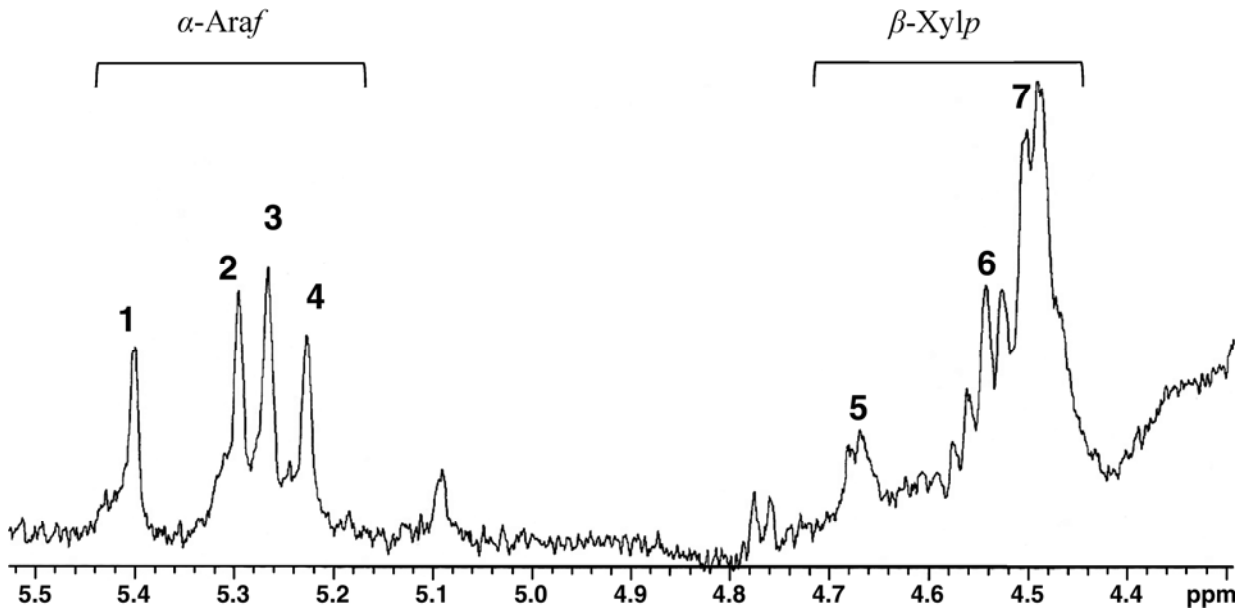

Fig. 1. Proton nuclear magnetic resonance spectrum of the water-extractable arabinoxylans of wheat bran. α-Ara*f* = α-L-arabinofuranosyl, and β-Xyl*p* = β-D-xylopyranosyl. Signals are identified as follows: 1 = X_1, anomeric proton of arabinose residue linked to the monosubstituted xylose at the O-3 position; 2 = X_2, anomeric proton of arabinose residue linked to the disubstituted xylose at the O-3 position; 3 = X_3, anomeric proton of arabinose residue linked to the disubstituted xylose at the O-2 position, when the xylose has an adjoining disubstituted xylose residue; 4 = X_4, anomeric proton of arabinose residue linked to the disubstituted xylose at the O-2 position; 5 = anomeric proton of disubstituted xylose residue; 6 = anomeric proton of monosubstituted xylose residue; and 7 = anomeric proton of nonsubstituted xylose residue. Resonance assignments were based on Hoffmann et al (1992).

Apparent Xylanase Activity

Table II lists the apparent xylanase activity level (hereafter referred to as xylanase activity) in wheat bran, flour, and whole meal in 12 different genotypes grown in three locations in 2008. In bran, the xylanase activity varied from 0.77 to 60.09 mU/g d.m. In flour, the variation was from 0.001 to 7.97 mU/g d.m. Gebruers et al (2010) found similar results for wheat flour and bran xylanase activities. The whole meal had a xylanase activity in the range of 0.12–31.78 mU/g d.m. Thus, xylanase activity was mainly concentrated in the wheat bran, whereas wheat flour contained low levels of xylanase. This general trend has been observed by other researchers (Gys et al 2004; Dornez et al 2006b; Gebruers et al 2010), who attributed most of the xylanase activity of grain to microbes residing mostly on the outer layers of the grain. Studies have shown that debranning of wheat kernels prior to milling helps reduce xylanase activity in wheat flour (Gys et al 2004). As xylanase plays an important role in wheat processing, reduction of xylanase levels can have a positive or negative effect, depending on the xylanase being removed and on the intended end use of the wheat. For example, in the case of breadmaking, reduction of the level of xylanases in wheat that preferentially hydrolyze WE-AX can be beneficial (Gys et al 2004). WE-AX are desirable in wheat flour breadmaking, as they help stabilize gas cells in dough (Courtin and Delcour 2002). Thus, wheat debranning prior to milling can have a positive effect on bread quality. On the other hand, debranning may reduce the level of xylanase with preference for WU-AX. This reduction will negatively affect WU-AX solubilization, which is preferred in breadmaking because WU-AX destabilize the gas cells in the dough (Courtin and Delcour 2002; Gys et al 2004).

The temperature and precipitation were relatively higher in Prosper than in Carrington during the 2008 growing season. This situation may have led to an increased microbial load in the Prosper samples. In our study, the xylanase activity level in bran, flour, and whole meal was highest in Prosper, followed by Casselton and Carrington (Table II). Among the HRSW genotypes, Ingot had the lowest xylanase activity (mean of 3.0 mU/g d.m.) and Granite the highest (mean of 37.3 mU/g d.m.) in bran for all the three growing locations. In the case of HWSW genotypes, the experimental line 99S0155-14W had the lowest mean xylanase activity (8.4 mU/g d.m.) and Lolo the highest (40.4 mU/g d.m.) in bran.

Analysis of variance indicated that there was a significant difference among locations, genotypes, and G × L interaction for xylanase activity levels in bran and whole meal ($P < 0.05$). Also, there was a significant difference between the HRSW and HWSW genotypes for xylanase activity ($P < 0.05$). The contribution of genotype, location and their interaction on xylanase activity in bran and whole meal was evaluated with variance component analysis (Fig. 2). About 40% of the variability in xylanase activity in bran and whole meal could be attributed to the effect of the location. This finding was similar to those by Gebruers et al (2010), who found 51% contribution from environment to xylanase activity in bran. Approximately 35% of the variability could be related to genotype, and about 17% of the variability came from G × L interaction. The relatively low contribution of genotype indicated that bran xylanase level was not a stable breeding parameter. However, Ingot and experimental line 99S0155-14W had a tendency toward low xylanase activity, whereas Granite and Lolo had a tendency toward high xylanase activity in all the three locations. Thus, these genotypes could be suitable candidates for further research.

The correlation between bran, flour, and whole meal xylanase activity with flour yield and bran ash percentage was calculated. There was no significant correlation (at $P < 0.001$) between bran, flour, and whole meal xylanase activity with flour yield or bran ash percentage. There was no significant correlation of bran, flour, and whole meal xylanase activity with flour yield. Thus, xylanase activity might show a better correlation with kernel characteristics such as 1,000-kernel weight or test weight rather than flour yield.

Apparent Xylanase Inhibition Activity of TAXI and XIP

Figures 3 and 4 show the TAXI and XIP inhibition activity levels in bran and flour. On average, the highest level of TAXI in the bran was seen in Alsen, whereas the lowest was for the Granite samples. However, in flour, Granite had the lowest levels of TAXI, but Alsen also had considerably low levels of TAXI. The average bran TAXI activity levels varied from 232.3 to 1,007.0 InU/g of d.m. For flour, TAXI activity varied from 11.95 to 274.13 InU/g of d.m. Thus, bran contained higher levels of TAXI than did flour (approximately a fivefold increase). Other studies have also shown higher levels of TAXI in bran than in flour (Gebruers et al 2010). Analysis of variance indicated bran TAXI levels had significant variance for genotypes and no significant variance for locations at a 5% level of significance. Thus, TAXI activity in wheat bran was mainly dependent on genotype. On the other hand, there was no significant difference between bran TAXI activity in the two classes of HRSW and HWSW at a 5% level of significance.

On average, the highest bran XIP levels were seen in the Ingot samples, whereas the lowest XIP levels were seen in the Granite

TABLE II
Apparent Xylanase Activity in Bran, Flour, and Whole Meal for 12 Hard Spring Wheat Genotypes[a]

	Apparent Xylanase Activity (mU/g d.m.)								
	Bran			Flour			Whole Meal		
Genotype	Carrington	Casselton	Prosper	Carrington	Casselton	Prosper	Carrington	Casselton	Prosper
Alsen[b]	2.69	14.93	18.08	0.02	0.19	0.38	0.38	3.25	6.17
Briggs[b]	1.77	6.03	16.67	0.02	0.12	0.65	0.12	1.06	6.86
Glenn[b]	0.95	12.89	16.00	0.00	0.05	0.31	0.89	2.19	4.59
Granite[b]	2.31	49.45	60.09	0.67	3.33	4.10	4.18	22.68	28.13
Hanna[b]	6.18	10.31	20.07	1.01	0.50	0.77	4.62	5.29	9.51
Ingot[b]	1.36	2.30	5.37	0.01	0.26	0.52	0.72	2.08	4.66
AC Snowbird[c]	7.26	10.51	29.79	2.08	1.27	2.80	7.47	8.23	15.43
Explorer[c]	4.71	27.02	37.88	0.34	2.06	3.87	3.60	16.02	25.32
Lolo[c]	5.47	58.40	57.33	1.11	7.97	7.05	3.62	31.78	30.99
CS3100Q[c]	9.72	33.97	46.63	3.05	2.70	3.34	6.43	7.46	28.30
Pristine[c]	0.77	46.92	44.97	0.94	3.78	3.70	4.58	22.77	26.10
99S0155-14W[c]	2.52	8.39	14.36	0.01	0.46	0.79	0.49	3.24	5.82
LSD	6.20	6.20	6.20	ns	1.28	0.96	0.70	0.70	0.70

[a] Grown at three different locations (Carrington, Casselton, and Prosper, North Dakota) in 2008. Data are mean values of two replications. LSD = least significant difference ($P < 0.05$); and ns = nonsignificant ($P > 0.05$).
[b] Hard red spring wheat.
[c] Hard white spring wheat.

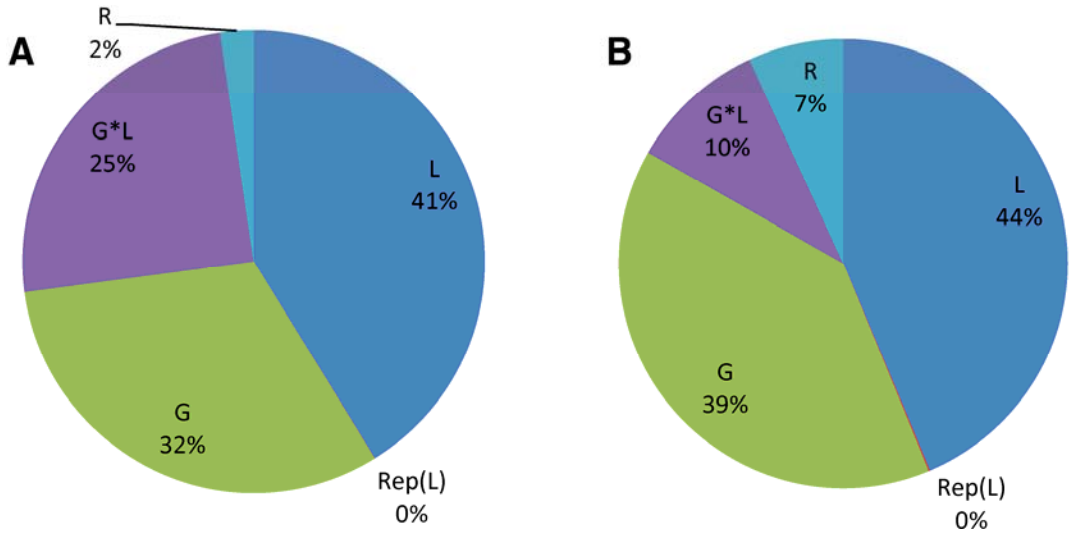

Fig. 2. Contribution (%) of genotype (G), location (L), interaction between G and L (G*L), replicates within L (Rep [L]), and the residual variability (R) to the apparent xylanase activity of wheat bran (**A**) and whole meal (**B**).

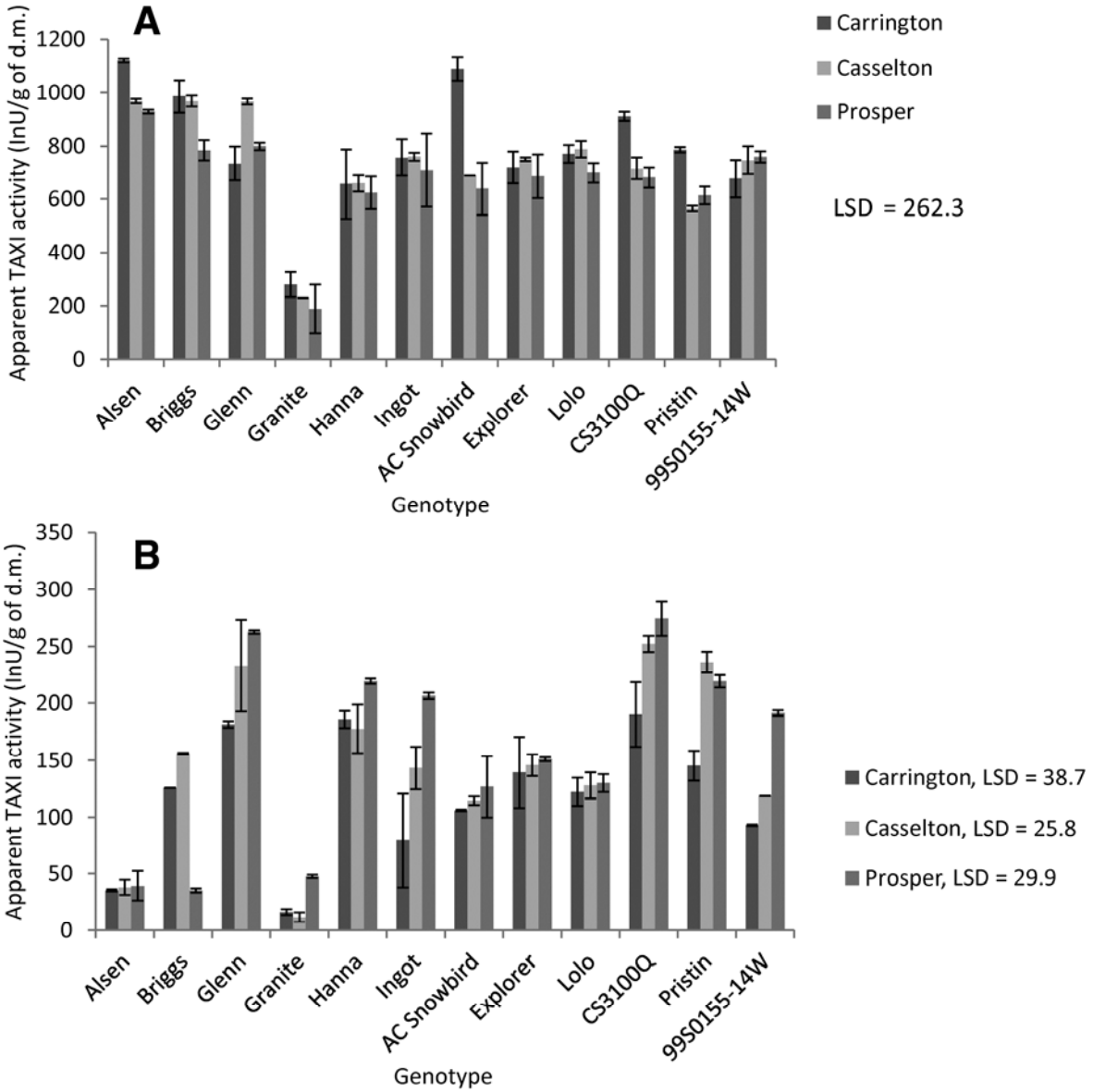

Fig. 3. Variability of *Triticum aestivum* xylanase inhibitor (TAXI) activity in bran (**A**) and flour (**B**) of different genotypes at different locations (Carrington, Casselton, and Prosper, North Dakota). InU = inhibition units; and LSD = least significant difference ($P < 0.05$) between genotypes within the same location.

samples (Fig. 4). However, XIP levels in flour did not follow this trend. In flour, the highest XIP levels were seen for the Alsen samples, whereas the lowest readings were observed for the Lolo samples. In all genotypes, bran contained higher levels of XIP than did flour (≈2.5-fold increase), in agreement with previously published data (Gebruers et al 2010). However, as also noted for TAXI, there was no significant difference between bran XIP activity in the two classes of HRSW and HWSW at a 5% level of significance.

Overall, XIP levels in bran were about three times higher than TAXI levels, in agreement with earlier data (Dornez et al 2006b, 2008). Flour XIP levels were approximately eight times higher than TAXI levels, whereas Bonnin et al (2005) for 20 French wheat cultivars observed only a threefold higher XIP level than TAXI level in flour. The occurrence of higher levels of xylanase inhibitors in the outer grain tissue layers of the wheat grain than in endosperm has been demonstrated (Dornez et al 2006a; Croes et al 2009). This spatial distribution of having high inhibitor levels in the outer layers of the grain suggested a role of TAXI and XIP in plant defense (Dornez et al 2010).

The contributions from each factor to TAXI activity (Fig. 5) and XIP activity in bran (Fig. 6A) and flour (Fig. 6B) were estimated. Genotype had the highest contribution to bran TAXI activity, accounting for approximately three-fourths of the variability (72%). Dornez et al (2008) reported that 77% of variability in TAXI in wheat whole grain was because of genotype. Based on the samples we tested, Granite had consistently very low TAXI levels in all three locations. The genotype contribution to the variability of XIP was relatively low. Our data suggested that ≈40% of variability in XIP activity (39% for bran and 44% for flour) was related to genotype. Gebruers et al (2010) observed 31% genotype contribution to variability in bran and flour XIP activity. For bran XIP, the contributions from G × L interaction and residual error were quite large (22% for G × L and 28% for residual). Others also reported large (≈10%) residual contributions for variability in XIP activity (Dornez et al 2008; Gebruers et al 2010). Igawa et al (2005) demonstrated that wheat xylanase inhibitor gene *Xip-I*, but not *Taxi-I*, was significantly induced by biotic and abiotic signals that triggered plant defense.

We calculated the correlation coefficient among parameters being tested in each location. There was no significant correlation among parameters in all three locations except for bran and flour xylanase and bran and whole meal xylanase. Different cereal-based processes have different preferences with respect to AX, xylanase, and xylanase inhibitors. Thus, the suitability of each genotype depends on the specific application. In dough and bakery products, xylanase can increase the elasticity and strength of the dough and act as a positive factor, whereas high levels of xy-

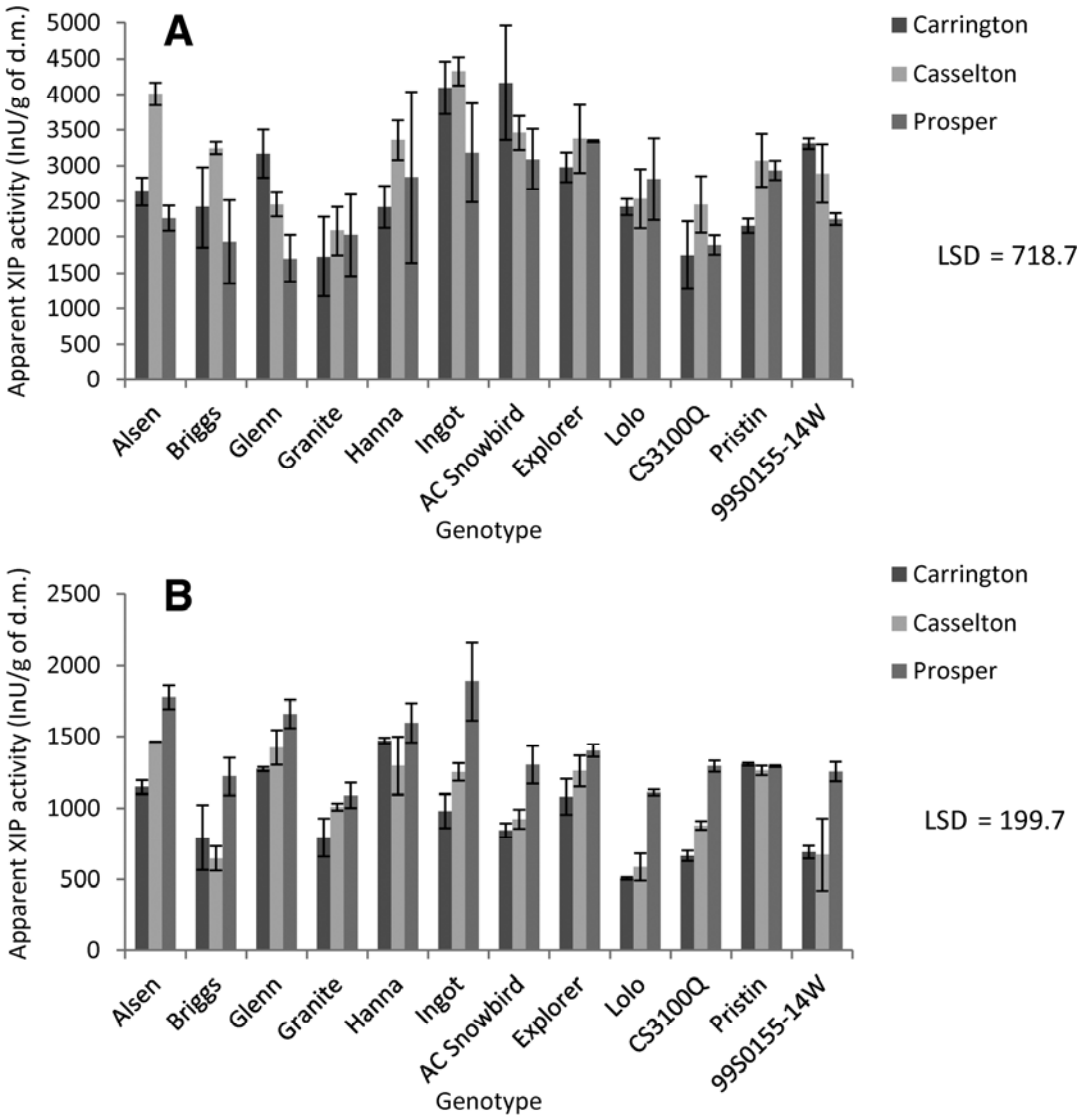

Fig. 4. Variability of xylanase inhibiting protein (XIP) activity in bran (**A**) and flour (**B**) of different genotypes at different locations (Carrington, Casselton, and Prosper, North Dakota). InU = inhibition units; and LSD = least significant difference ($P < 0.05$) between genotypes within the same location.

lanase negatively affect pasta production and the shelf life of refrigerated dough (Collins et al 2005; Dornez et al 2009). High levels of AX negatively affect gluten–starch separation. Thus, genotypes with high xylanase and low AX levels are preferred in such applications. Microbial xylanases are routinely used in many cereal-processing industries to improve processing and end-product quality. However, the net effect of these added xylanases can be affected by the xylanase inhibitors present in the grain. Because different xylanases have different specificities to TAXI and XIP, our data on the levels of TAXI and XIP can help the industry in deciding the type of xylanase to be added to achieve its maximum effect with minimal inhibition from the inhibitor. We calculated the correlation of milling yield and bran ash to each of the parameters and could not find a significant correlation (at $P < 0.001$) between bran TAXI, bran XIP, flour TAXI, or flour XIP with flour yield or bran ash. Thus, based on our sample, the xylanase inhibitor activity was not significantly affected by milling yields.

CONCLUSIONS

We here studied the variability of AX level, xylanase activity, and xylanase inhibition activity in 12 hard spring wheat genotypes consisting of six HRSW and six HWSW classes, in relation to the effect of genotype, location, and G × L interaction. There was no significant difference in TOT-AX among the bran of 12 genotypes (at $P < 0.05$). However, there was a significant difference in mean values of bran TOT-AX between the two wheat classes, HRSW and HWSW ($P < 0.05$). Overall, HWSW bran had higher TOT-AX than HRSW bran. Within our sample set, Explorer had the highest TOT-AX in bran (27.96%). There were significant differences in the A/X ratio of the brans among genotypes ($P < 0.05$), which indicated that variations in substitution pattern in AX were genotype dependent. We analyzed bran WE-AX with ^{1}H-NMR. Because of the possible contamination of bran with starchy endosperm, bran WE-AX also contained endosperm WE-AX. Based on the ^{1}H-NMR results, Ingot and Lolo indicated a high level of disubstitution along the xylan backbone. The overall spectral data indicated that the substitution pattern favored disubstitution over monosubstitution.

The xylanase activity was mainly concentrated in the wheat bran, with the highest average xylanase activity in HWSW genotype Lolo. The lowest average xylanase activity was seen in HRSW genotype Ingot. We calculated the contribution from each factor to xylanase activity in wheat bran and whole meal. Approximately 35% of the variability could be related to genotype, and 17% of the variability came from G × L interaction. The relatively high level of the G × L interaction term indicated that bran xylanase level was not a stable breeding parameter.

TAXI and XIP levels were higher in bran than in flour, suggesting that these inhibitors were concentrated in the outer layers of the grain. Overall, XIP levels in bran and flour were about three and eight times higher than TAXI levels, respectively. Genotype greatly contributed to the variability of TAXI activity specifically, showing 72% contribution to that in wheat bran. For variability of XIP activity, the genotype contribution was smaller than for TAXI, showing 39 and 44% for bran and flour, respectively. These results indicated that the bran TAXI activity levels were largely under genetic control, and breeders can aim at achieving high or low TAXI levels as a parameter influencing xylanase activity in hard spring wheat.

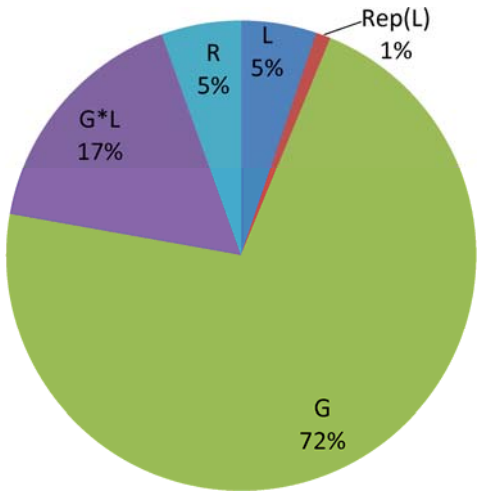

Fig. 5. Contribution (%) of genotype (G), location (L), interaction between G and L (G*L), replicates within L (Rep [L]), and the residual variability (R) to the variation in apparent *Triticum aestivum* xylanase inhibitor activity of wheat bran.

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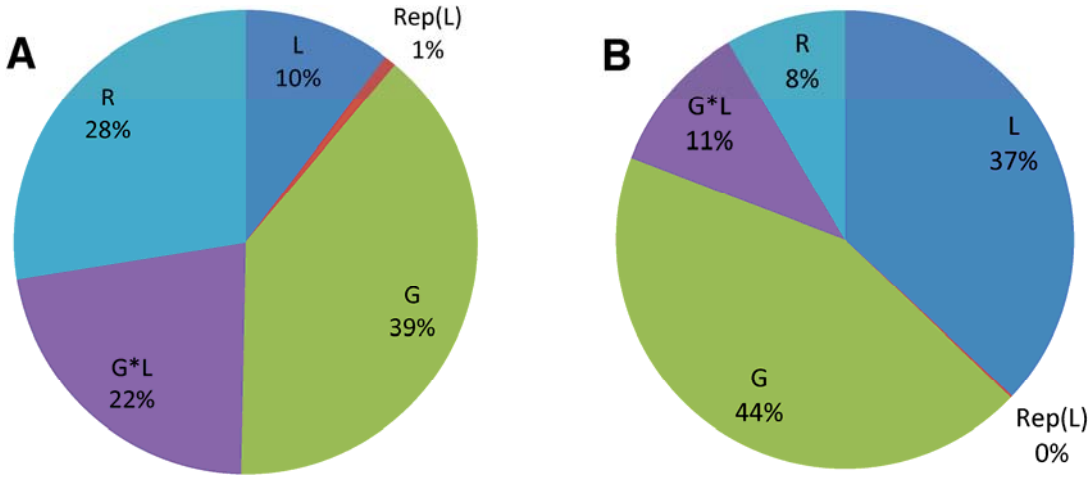

Fig. 6. Contribution (%) of genotype (G), location (L), interaction between G and L (G*L), replicates within L (Rep [L]), and the residual variability (R) to the variance in apparent xylanase inhibiting protein activity of wheat bran (**A**) and flour (**B**).

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