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ORIGINAL PAPER

Prevalence of *Puroindoline D1* and *Puroindoline b-2* variants in U.S. Pacific Northwest wheat breeding germplasm pools, and their association with kernel texture

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Abstract Kernel texture is a major factor influencing the classification and end use properties of wheat (*Triticum aestivum* L.), and is mainly controlled by the *Puroindoline a* (*Pina*) and *Puroindoline b* (*Pinb*) genes. Recently, a new puroindoline gene, *Puroindoline b-2* (*Pin b-2*), was identified. In this study, 388 wheat cultivars and advanced breeding lines from the U.S. Pacific Northwest were investigated for frequencies of *Puroindoline D1* alleles and *Pinb-2* variants 2 and 3. Results indicated that *Pinb–D1b* (74.0%) was the predominant genotype among hard wheats ($N = 196$), the only other hard allele encountered was *Pina-D1b* (26.0%). Across all varieties, *Pinb-2v3* was the predominant genotype (84.5%) compared with *Pinb-2v2* (15.5%). However, among 240 winter wheat varieties (124 soft white, 15 club, 68 hard red and 33 hard white varieties), all carried *Pinb-2v3*. Among spring wheats, *Pinb-2v2* and *Pinb-2v3* frequencies were more variable (soft white 25.0:75.0, hard red 58.2:41.8 and hard white 40.0:60.0, respectively). Kernel texture variation was analyzed using 247 of the 388 wheat varieties grown in multi-location factorial trials in up to 7 crop years. The range of variety means among the four groups, soft winter, soft spring, hard winter and hard spring, was on the order of 15–25 single kernel characterization system (SKCS) Hardness Index. The least significant difference for each of these trials ranged from 2.8 to 5.6 SKCS Hardness Index. Observations lead to the conclusion that *Pinb-2* variants do not exert a prominent effect on kernel texture, however, *Pinb–2* variants do identify features of wheat germ plasm structure in the U.S. Pacific Northwest.

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Introduction

Kernel texture (grain hardness) is an important end-use quality trait in wheat (*Triticum* sp. L). Among the primary wheats of commerce, three kernel texture classes are recognized: soft and hard hexaploid (*T. aestivum*), and very hard durum (*T. turgidum* ssp. *durum*). The genetic basis for these classes is well established and can be explained by the presence, absence or gene sequence of the puroindolines, 'a' and 'b' (Pina, Pinb), coded at the *Pina-D1* and *Pinb-D1* loci on the distal end of the short arm of chromosome 5D (Morris 2002; Bhave and Morris 2008a, b). Whereas, soft wheats

carry the 'wild-type' puroindoline haplotype (designated *Pina–D1a/Pinb–D1a*), hard wheats have been shown to carry a lesion in *Pina*, *Pinb* or both (Bhave and Morris 2008a, b; Morris and Bhave 2008) resulting in a "loss or diminished function". Giroux and Morris (1997, 1998) documented the first two hardness mutations in wheat, *Pina-D1b* and *Pinb-D1b*. Since these gene sequence polymorphisms are hypothesized to have arisen by mutation post-hexaploidization, their occurrence/prevalence is in part indicative of germplasm structure, movement and founder effects. The puroindolines occur in apparent unbroken linkage haplotypes, for e.g., *Pina-D1a/Pinb-D1a*, *Pina-D1b/Pinb-D1a* and *Pina–D1a/Pinb-D1b*. The first confers soft kernel texture, the latter two hard kernel texture.

The following studies documented germplasm and variety surveys, some provided single kernel characterization system (SKCS) kernel texture of various puroindoline haplotypes. Puroindoline allele frequencies were reported among wheat varieties from Europe, US, Canada and Latin American (Lillemo and Morris 2000), North America (Morris et al. 2001), India and Kansas (Ram et al. 2002), Portugal (Bagulho et al. 2003), southern Australia (Cane et al. 2004), Europe (Huang and Röder 2005), Chinese breeding programs (Xia et al. 2005), China (Chang et al. 2006, Chen et al. 2006, 2010b), CIMMYT (Lillemo et al. 2006), Asia (Tanaka et al. 2008), and various other variety collections (Ikeda et al. 2005; Ravel et al. 2006; Chen et al. 2007; Pickering and Bhave 2007). In total, seven *hardness* alleles were found (*Pina-D1b*, and *Pinb-D1b, c, d, e, f, p*). By far, the most frequent were *Pina-D1b* and *Pinb-D1b*. In general, varieties with the *Pina-D1b* allele were found to have a harder SKCS value compared to *Pinb-D1b* (Lillemo and Morris 2000, 75 vs. 68; Xia et al. 2005, 76 vs. 66; Chen et al. 2006, 70 vs. 62; Lillemo et al. 2006, 88 vs. 80; Chen et al. 2007, 69 vs. 59 and 74 vs. 65).

A few studies took a more genetically controlled examination of the role of puroindoline hardness alleles in kernel texture. Giroux et al. (2000) examined three hard by hard wheat crosses. On average, lines with the *Pina-D1b* allele were harder than lines with *Pinb-D1b*. Yet, they concluded, "that most of the genetic variation in grain hardness among the populations studied was due to factors other than *pinA* [*sic Pina*] and *pinB* [*Pinb*], as the *PinA-D1b* versus *PinB-D1b* differences explained <12% of the variation in grain hardness". Martin et al. (2001) examined RILs from a hard spring by hard spring cross (*Pina-D1b* by *Pinb-D1b*). Mean kernel texture by puroindoline haplotype was 64.3 versus 58.3, *Pina-D1b* and *Pinb-D1b*, respectively. The narrow sense heritability for SKCS kernel texture was 0.88, the range among RILs was 41.8–73.6, with a CV of 3.6%. The parents differed by only 2.8 units (61.0 vs. 63.8, not significant). The proportion of variation among means attributable to the difference between *Pina-D1b* and *Pinb-D1b* was only 22% for SKCS kernel texture. Martin et al. (2008) later studied a second RIL population developed from the two hard spring wheats (*Pina-D1b* by *Pinb-D1b*). SKCS kernel texture was significantly different between the two RIL haplotype groups (68.9 vs. 74.5, *Pinb-D1b* vs. *Pina-D1b*). Ma et al. (2009) examined seven BC_7 puroindoline haplotype near isogenic lines (NILs) developed in the soft white spring wheat cultivar Alpowa background (Morris and King 2008). The *Pina-D1b* NIL was significantly harder than the *Pinb-D1b* NIL (75.5 vs. 69.6).

Recently, a new *Puroindoline b* gene was reported. Wilkinson et al. (2008) obtained 172 ESTs from Genbank related to *Pinb-D1*. Most (95%) were *Pinb-D1a*. Variant sequences were denoted *Pinb-2* 'variant 1', 'variant 2', and 'variant 3'. cDNAs were isolated from cv. Hereward for variants 1 and 2, but not 3. ESTs related to variant 3 were obtained from *T. aestivum* cvs. Mercia and Cheyenne. PCR of mRNA from the durum cv. Ofanto amplified variant 3, but not 1 or 2. However, PCR on genomic DNA of Ofanto produced variants 1 and 3. Variant 1 was mapped to 7AL in a DH population; they designated the locus *Pinb-A2*. Two additional DH populations were also examined. Although the parents of each cross were polymorphic for the *Pinb-D1* and *Pinb-2* loci, no kernel texture QTLs consistently mapped to either locus. *Pinb-2* variant 1 was mapped to 7AL in all three crosses. They indicated that the *Pinb-2* variants were expressed at probably less than 10% of the levels of transcripts encoding *Pina-D1* and *Pinb-D1*.

Chen et al. (2010a) isolated 43 *Pinb-2* clones, which all matched perfectly the existing variant 1, 2 and 3 sequences of Wilkinson et al. (2008); additional 5′ flanking sequence was obtained. Using degenerate primers, a second group of clones were obtained; 85% were *Pina* or *Pinb*, the remainder had a novel sequence which was designated 'variant 4.' Sequencing provided the *Pinb-2* haplotype for Chinese Spring and four other varieties. All possessed variants 1 and 4, whereas only variant 2 or 3 was present. A combination of Chinese Spring ditelosomic lines, group 7 chromosome nullisomic–tetrasomic lines, and disomic substitution lines with Cheyenne showed that the *Pinb-2* variants physically mapped to chromosomes 7DL (*Pinb-2v1*), 7BL (*Pinb-2v2*), 7B (*Pinb-2v3*), and 7AL (*Pinb-2v4*). *Pinb-2v2* was not detected in Cheyenne, whereas *Pinb2-v3* was not detected in Chinese Spring (Chen et al. 2010a, 2011).

In a recent follow-up study, Chen et al. (2010b) examined the frequency of *Pinb-2* alleles among varieties from the Yellow and Huai Valleys of China. The complete set of 169 was comprised of 131 current popular varieties and 38 landraces. Based on SKCS these were classified as 57 soft, 15 mixed and 97 hard. All of the soft and hard varieties ($n = 154$) possessed *Pinb-2* variants 1 and 4 (mixed varieties were not tested), with 37 *Pinb-2v2* (variant 2) and 117 *Pinb-2v3* (variant 3). Analysis of variance (ANOVA) was

conducted on SKCS kernel texture within each *Pin-D1* haplotype class. The effect of *Pinb-2v2* versus *v3* was declared significant ($P < 0.01$) only in the soft class. Means of the two groups were 22.2 and 27.2, *v2* and *v3*, respectively. In the *Pina-D1b* and *Pinb-D1b* hard haplotype groups the contrast was not significant.

The present report re-examines the possible role of the *Puroindoline b-2* gene variants in wheat kernel texture in a regional wheat gene pool largely unrelated to previous studies on *Pinb-2*. Released cultivars and advanced breeding lines were collected from 7 years of testing and included soft winter, soft spring, hard winter and hard spring types. In addition, soft white club, and red and white hard grained varieties were included. All germplasm were haplotyped for *Pina-D1* and *Pinb-D1*, and for the *Pinb-2* variants 2 and 3. Phenotypic SKCS kernel texture data were obtained from 7 crop years with 3 to 7 locations per year. The study was divided into two parts, the first (Part I) deals with haplotype frequency among 388 varieties, the second (Part II) deals with the multi-environment SKCS phenotypic data and possible associations with *Puroindoline b-2* variants.

Materials and methods

Wheat germplasm and kernel texture phenotyping

For Part I, 388 unique wheat cultivars and advanced breeding lines from the U.S. Pacific Northwest were evaluated for the prevalence of *Puroindoline-D1* and *Puroindoline b-2* variants (Table 1). Of the 388 varieties, 313 were obtained from ongoing "Genotype and Environment" (G&E) studies conducted by the U.S. Department of Agriculture, Agricultural Research Service, Western Wheat Quality Laboratory (WWQL), and were grown in Washington, Oregon and/or Idaho during one or more crop years from 2004 to 2010 (indicates year of harvest for spring and winter wheats). The remaining 75 varieties were grown as part of the Western Regional Nursery harvested in 2010. Duplicate varieties appearing in more than one nursery or crop year were eliminated for this portion of the research (Part I). The most advanced, and therefore promising, breeding lines were included in the nurseries comprising the Washington, Oregon and Idaho G&E studies, and the Western Regional Nurseries. The G&E nurseries also included most of the commercial cultivars available to growers.

In Part II, ANOVA of kernel texture phenotype, and the relationship between *Puroindoline-D1* alleles, *Puroindoline b-2* variants and kernel texture were assessed among a subset of 247 unique varieties grown in multiple environments from the aforementioned Washington G&E study (Table 1). Seeding, plot management and harvesting were conducted by the Washington State University Cereal Variety Testing program. All of these varieties were haplotyped for *Pin-D1* and *Pinb-2* in Part I. In total, 2,812 grain samples were included. Kernel texture (hardness), kernel weight and kernel diameter were measured on 300-kernel samples using the Perten SKCS 4100, following the manufacturer's operating procedure (Perten Instruments North America Inc., Springfield, IL, USA).

Table 1 Nursery identifier, classification, and the number of wheat cultivars and advanced breeding lines included in this study

Nursery[a]	Classification	Number of varieties
Washington G&E	SWW, club	79
	SWS, club	39
	HRW, HWW	63
	HRS, HWS	66
	Subtotal	247
Oregon G&E	SWW, club	25
	SWS, club	0
	HRW, HWW	28
	HRS, HWS	0
	Subtotal	53
Idaho G&E	SWW, club	2
	SWS, club	0
	HRW, HWW	2
	HRS, HWS	8
	Subtotal	13
Western regional	SWW, club	33
	SWS, club	14
	HRW, HWW	8
	HWS	20
	Subtotal	75
Total		388

SWW soft white winter, *Club* soft white club, *SWS* soft white spring, *HRW* hard red winter, *HWW* hard white winter, *HRS* hard red spring, *HWS* hard white spring wheat varieties

[a] *G&E* genotype and environment study nurseries analyzed at the WWQL

Detection of *Puroindoline-D1* alleles and *Puroindoline b-2* variants

Wheat genomic DNA was extracted from leaf tissue of two 10-days-old individual seedlings according to the procedures described by Riede and Anderson (1996). Giroux and Morris (1998) established that varieties that lack the PINA protein (*Pina-D1b*) have a 'soft' wild-type PINB protein (coded by *Pinb-D1a*); these varieties will simply be referred to as *Pina-D1b*. Those having the glycine-to-serine translated mutation conversely have a soft wild-type PINA protein (coded by *Pina-D1a*); these varieties will be referred to as *Pinb-D1b*. In this study, the *Puroindoline*

Table 2 PCR primers used in generating *Puroindoline b-2* variant gene sequences in wheat

Gene	Forward primer	Reverse primer	PCR annealing temperature (°C)	Fragment size (bp)
Pina-D1b	ACAACCGCACACAGAAATCG	CAATGGGCGCCACTATAACA	60	326
Pinb-D1b	TCACCAGTAATAGCCAATAGTG	ATGAAGGCCCTCTTCCTCA	60	447
Pinb-2v2	CTTGTAGTGAGCACAACCTTTGCA	GTATGGACGAACTTGCAGCTGGAG	65	401
Pinb-2v3	GAGCACAACCTTTGCGCAATG	CATTAGTAGGGACGAACTTGCAGCTA	65	398

allele *Pina-D1a* was amplified with the forward primer 5′TCACCAGTAATAGCCAATAGTG3′ and reverse primer 5′ATGAAGGCCCTCTTCCTCA3′ yielding an expected PCR product of 447 bp. The other *Puroindoline* allele, *Pina-D1b*, was amplified with the forward primer 5′ACAACCGCACACAGAAATCG3′ and reverse primer 5′CAATGGGCGCCACTATAACA3′ yielding an expected PCR product of 326 bp (Table 2). *Puroindoline b–2* variant 2 (*Pinb-2v2*) and variant 3 (*Pinb-2v3*) were identified using variant specific primers (Table 2) according to the methods reported by Chen et al. (2010a).

PCRs were performed in an MJ Research PTC-200 thermal cycler in a total volume of 25 μL including 250 μM of each dNTP, 10 pmol of each primer, 100 ng of gDNA, 1× reaction buffer (50 mmol of KCl, 10 mmol of Tris–Cl, 1.5 μM of $MgCl_2$, pH 8.4) and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR conditions were 94°C for 5 min, followed by 45 cycles of 94°C for 50 s, 60–65°C for 50 s (for primer-specific annealing temperatures, see Table 1) and 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels. The bands were stained with ethidium bromide and visualized using UV light.

Statistical analysis

Analysis of kernel texture data was carried out using SAS version 9.2 (SAS Institute, Cary, NC, USA). Procedure general linear model (PROC GLM) was used for generating ANOVA tables using Type III sums of squares. A genotypic variance ratio, σ^2, was calculated using ANOVA Type III sums of squares (sample variance, s^2) as follows:

$$\left\{\left[\left(s_G^2/g\right)\right]/\left[\left(s_G^2/g\right)+\left(s_L^2/l\right)+\left(s_E^2/\varepsilon\right)\right]\right\}\times 100,$$

where s_G^2, s_L^2 and s_E^2 are the genotype (i.e., variety), location and error variances, respectively, g and l are the number of varieties and locations, respectively, and ε is the error degrees of freedom plus one. This genotypic variance ratio essentially describes the percentage of variation assignable to varieties over the total variation of the experiment, adjusted for the variable number of varieties and locations that appear in each class and crop year. Simple (Pearson) correlation coefficients, r, among kernel texture traits were also computed by SAS software.

Results

Frequency of *Puroindoline-D1* alleles in Pacific Northwest wheat germplasm

Of the 388 wheat varieties included in the present study, 196 were hard wheat varieties, which included 68 hard red winter, 33 hard white winter, 40 hard white spring, and 55 hard red spring. All were haplotyped using *Puroindoline-D1* allele-specific primers. Of the 196 hard wheat samples, 26.0% had the PINA-null mutation, *Pina-D1b*, whereas the other 74.0% possessed the *Pinb-D1b* allele (Table 3). No other *hardness* mutations were encountered. Although this overall ratio was about 1:3, the relative frequencies of *Pina-D1b* and *Pinb-D1b* among the four hard wheat groups were quite different. Among the 68 hard red winter wheats, all but four (94.1%) carried *Pinb-D1b* (Table 3). A similar frequency was observed among the 33 hard white winter wheats (90.9%, all but three). For hard red spring wheat, the frequencies of *Pina-D1b* and *Pinb-D1b* genotypes were 56.4 and 43.6%, respectively, and for hard white spring wheat, 32.5% and 67.5%, respectively. These results indicated that across all hard wheat types, the *Pinb-D1b* was the predominant allele. Compared with winter wheat, the frequencies of *Pinb-D1b* in the red white and white spring wheat types were much lower, being nearly equal in hard red spring. It should be noted that all soft wheats carry the *Pina-D1a/Pinb-D1a* haplotype.

Frequency of *Puroindoline b-2* variants in Pacific Northwest wheat germplasm

The same 388 wheat varieties (Table 1) were investigated for *Puroindoline b-2* variants. Based on results with *Puroindoline b-2* variant-specific primers, 60 varieties (15.5%) possessed *Pinb-2v2* (variant 2), whereas the other 328 varieties (84.5%) possessed *Pinb-2v3* (variant 3) (Table 3). Broken down by type, of note, among the 240 winter wheat varieties, which included 124 soft white winter, 15 soft white club, 68 hard red winter, and 33 hard

Table 3 Frequencies of *Puroindoline b-2* variants in eight groups of U.S. Pacific Northwest wheat varieties

Classification	No. of samples	*Puroindoline D1* alleles[a]		*Puroindoline b-2* variants	
		Pina-D1b (%)	*Pinb-D1b* (%)	*Pinb-2v2* (%)	*Pinb-2v3* (%)
Soft white winter (SWW)	124	–	–	0.0	100.0
Soft white club (Club-winter)	15	–	–	0.0	100.0
Soft white spring (SWS)	44	–	–	25.0	75.0
Soft white club (Club-spring)	9	–	–	11.1	88.9
Hard red winter (HRW)	68	5.9	94.1	0.0	100.0
Hard white winter (HWW)	33	9.1	90.9	0.0	100.0
Hard red spring (HRS)	55	56.4	43.6	58.2	41.8
Hard white spring (HWS)	40	32.5	67.5	40.0	60.0
Subtotal, hard	196	26.0	74.0	24.5	75.5
Total	388	–	–	15.5	84.5

[a] All soft wheats carry the *Pina-D1a/Pinb-D1a* haplotype; each hard wheat variety carries one mutation in either *Pina* or *Pinb*

white winter, all (100%) carried *Pinb-2v3* (Table 3). For soft white spring wheat, the frequencies of *Pinb-2v2* and *Pinb-2v3* genotypes were 25.0 and 75.0%, respectively; for hard red spring wheat 58.2 and 41.8%, respectively, and for hard white spring wheat 40.0 and 60.0%, respectively. Of the 24 soft white club wheat varieties (winter and spring), all but one (95.8%) carried *Pinb-2v3*. Compared with the other types, the hard red spring wheat group again had the greatest difference in *Puroindoline b2* allele frequency. In the U.S. grain grading system, winter and spring club wheats are not delineated in classification. The sole club wheat variety possessing *Pinb-2v2* was the spring type breeding line, WA8082. Therefore, all of the 60 varieties with the *Pinb-2v2* allele were exclusively spring wheats (across all spring wheats, 40%). These results indicated, however, that *Pinb-2v3* was the preponderant allele in Pacific Northwest germplasm.

Correlations among kernel characteristics

Kernel characteristics, particularly kernel weight and size, are important components of grain yield and quality in wheat (Tsilo et al. 2010). To see if these traits had any relationship to kernel texture, the correlation coefficients, *r*, were calculated among the following parameters: kernel texture, kernel weight and kernel diameter (data not shown). For each of the four wheat groups based on winter-spring and soft-hard kernel combinations, kernel texture was significantly negatively correlated with kernel weight (soft winter wheat, $r = -0.19$; soft spring wheat, $r = -0.53$; hard winter wheat, $r = -0.44$; hard spring wheat, $r = -0.60$; all at $P < 0.0001$), and variably correlated with kernel diameter (soft winter wheat, $r = -0.04$, $P = 0.16$; soft spring wheat, $r = -0.38$, and hard spring wheat, $r = -0.48$, both at $P = < 0.0001$; hard winter wheat, $r = -0.11$, $P = 0.0045$). Kernel weight showed a positive relationship with kernel diameter for these four groups $r = 0.59$–0.82, $P < 0.0001$). These results indicated that harder kernel texture had some relationship with lower kernel weight and smaller kernel diameter. Similar results were obtained by Martin et al. (2001) who also found modest negative correlations between kernel texture and kernel weight ($r = -0.28$, $P < 0.01$), and kernel diameter $r = -0.21$, $P < 0.05$). Tsilo et al. (2010) similarly found a positive relationship between kernel weight and kernel diameter ($r = 0.93$, $P < 0.001$).

Analysis of phenotypic variance and genotypic contribution to kernel texture variation

The 247 varieties included in the Washington G&E study (Table 1) were evaluated for kernel texture variation from multiple environments within each of 7 crop years. Variation in kernel texture was analyzed based on the four winter–spring, soft–hard groups as this is how they were grouped and grown in the various environments. All of the analyses were conducted on a crop year basis with a completely balanced factorial design wherein all varieties appeared at all locations. This portion of the study included 79 soft winter, 39 soft spring, 63 hard winter, and 66 hard spring wheat varieties (Table 1). In total, 2,812 grain samples were analyzed (four of the possible 2,816 data points not included). Due to the nature of advanced yield testing, the composition of each nursery in terms of varieties varied from year to year (as did location), but often a specific breeding line would appear for several consecutive years; cultivars more frequently. In most instances, nurseries were grown at five Washington locations each year (Tables 4, 5, 6, 7). For all four groups, the ANOVA models appeared robust in that they explained 69–93% of the total variation in kernel texture across each of the seven crop years (2004–2010). The *F* values for the whole model, and variety and location model components were all highly significant ($P < 0.001$), and indicated roughly similar variation for kernel texture among all four groups. Often, the *F* value for location was considerably greater than variety (but not consistently so),

Table 4 Number of varieties and locations, variety mean and range, ANOVA whole model R^2 and F values, variety and location F values, the ANOVA model least significant difference (LSD) for variety mean separation, and a genotypic variance ratio for SKCS kernel texture of U.S. Pacific Northwest soft winter wheat varieties harvested in 2004–2010 from the WSU Cereal Variety Testing Program

Source	2004	2005	2006	2007	2008	2009	2010
Variety number	21	23	35	22	27	31	19
Location number	6	6	6	5	5	5	7
Variety mean	30.6	30.9	41.2	32.9	24.2	27.5	28.1
Variety range	13.6–41.6	12.6–42.7	27.5–52.4	17.3–43.5	14.7–33.7	11.3–38.2	20.4–35.5
Whole model R^2	0.92	0.90	0.85	0.93	0.82	0.92	0.79
Whole model F value	49.1***	37.7***	24.2***	44.5***	15.5***	40.6***	16.7***
Variety F value	28.1***	23.6***	24.8***	41.1***	11.2***	23.1***	11.8***
Variety LSD	3.5	3.7	3.9	3.0	4.7	3.7	3.5
Location F value	133.0***	99.4***	19.7***	62.7***	42.9***	172.2***	31.6***
σ^2 ratio	19.3	21.2	58.1	43.4	23.5	13.8	28.4

*** $P < 0.001$

Table 5 Number of varieties and locations, variety mean and range, ANOVA whole model R^2 and F values, variety and location F values, the ANOVA model least significant difference (LSD) for variety mean separation, and a genotypic variance ratio for SKCS kernel texture of U.S. Pacific Northwest soft white spring wheat varieties harvested in 2004–2010 from the WSU Cereal Variety Testing Program

Source	2004	2005	2006	2007	2008	2009	2010
Variety number	8	7	10	15	16	19	16
Location number	6	5	5	5	5	7	5
Variety mean	22.1	36.1	35.1	26.6	24.2	20.9	25.8
Variety range	16.7–29.8	27.5–46.8	28.4–47.6	21.1–34.5	18.1–36.2	14.0–31.1	19.8–36.0
Whole model R^2	0.88	0.86	0.83	0.84	0.84	0.79	0.89
Whole model F value[a]	22.2***	14.9***	13.9***	15.8***	16.9***	17.1***	26.7***
Variety F value	17.1***	18.5***	15.0***	6.5***	13.0***	15.8***	15.2***
Variety LSD	3.3	4.9	4.5	4.8	3.9	3.2	3.5
Location F value	29.2***	9.6***	11.4***	48.3***	31.6***	20.9***	68.5***
σ^2 ratio	37.2	64.6	57.2	13.3	31.6	44.2	20.8

*** $P < 0.001$

Table 6 Number of varieties and locations, variety mean and range, ANOVA whole model R^2 and F values, variety and location F values, the ANOVA model least significant difference (LSD) for variety mean separation, and a genotypic variance ratio for SKCS kernel texture of U.S. Pacific Northwest hard winter wheat varieties harvested in 2004–2010 from the WSU Cereal Variety Testing Program

Source	2004	2005	2006	2007	2008	2009	2010
Variety number	10	11	25	22	23	28	13
Location number	6	3	5	5	5	5	5
Variety mean	66.8	67.9	75.5	70.7	73.5	71.4	73.3
Variety range	55.8–78.8	53.2–74.4	59.5–85.3	56.8–79.9	66.8–82.3	62.0–79.3	65.6–78.9
Whole model R^2	0.79	0.92	0.82	0.82	0.82	0.87	0.69
Whole model F value	12.0***	18.9***	15.5***	15.1***	14.9***	21.8***	6.5***
Variety F value	12.1***	15.3***	10.9***	10.7***	14.1***	15.7***	7.1***
Variety LSD	5.6	4.6	4.8	4.6	2.8	3.5	4.9
Location F value	12.1***	37.3***	42.8***	37.9***	19.7***	65.6***	5.0**
σ^2 ratio	49.5	35.0	22.9	24.7	44.5	22.1	56.9

** $P < 0.01$; *** $P < 0.001$

Table 7 Number of varieties and locations, variety mean and range, ANOVA whole model R^2 and F values, variety and location F values, the ANOVA model least significant difference (LSD) for variety mean separation, and a genotypic variance ratio for SKCS kernel texture of U.S. Pacific Northwest hard spring wheat varieties harvested in 2004–2010 from the WSU Cereal Variety Testing Program

Source	2004	2005	2006	2007	2008	2009	2010
Variety number	12	11	25	26	24	24	10
Location number	5	5	5	5	5	5	5
Variety mean	70.3	71.0	74.9	67.7	64.3	59.3	65.2
Variety range	62.4–75.4	63.2–77.1	60.8–92.6	56.3–83.1	52.1–81.5	47.6–78.8	59.9–75.7
Whole model R^2	0.83	0.83	0.93	0.89	0.93	0.90	0.89
Whole model F value	14.6***	14.1***	49.0***	29.4***	43.0***	31.0***	23.1***
Variety F value	10.4***	9.9***	38.9***	22.1***	25.7***	28.3***	11.8***
Variety LSD	3.6	3.8	3.1	4.1	4.0	3.9	4.4
Location F value	26.2***	24.6***	109.4***	74.7***	142.3***	46.7***	48.4***
σ^2 ratio	30.3	30.4	29.7	25.9	17.7	41.4	21.2

*** $P < 0.001$

suggesting that the environment (viz. location) had a pronounced effect on kernel texture, but that the effect was relatively consistent across varieties. The ANOVA kernel texture least significant difference (alpha = 0.05) for all 28 separate analyses fell within a fairly narrow range of 2.8–5.6 SKCS Hardness Index, against a range of class-year means of 20.9–41.2 for soft wheats, and 59.3–77.5 for hard wheats. These results indicate the relatively high precision of kernel texture measurement and heritability among wheat varieties. In this regard, we calculated a genotypic variance ratio (σ^2) for each class-year. This ratio (expressed as a percentage) evidenced the genotypic contribution to total variation within a crop year, wherein the variety, location and error variances were adjusted according to the number of observations.

Across all 28 wheat group–crop year combinations, the genotypic variance ratio for SKCS kernel texture ranged from 13.3 to 64.6% (Tables 4, 5, 6, 7). There did not appear to be a particular pattern associated with wheat group, crop year or the overall ANOVA model R^2. However, not too surprising, the ratio did track somewhat the variety versus location F value ratios, independent of the variety F value absolute value (Tables 4, 5, 6, 7). Although not directly applicable to estimating heritability, this variance ratio does illustrate the range of genetic contribution that can be encountered in multi-environment trials of non-segregating advanced germplasm, and in conjunction with the LSDs, an indication as to what level of genetically-determined kernel texture (SKCS Hardness Index) might be selected for.

Association of *Pin-D1* alleles and *Pin b-2* variants with kernel texture

In this study, allele-specific PCR showed that all of the hard varieties had either the *Pina-D1b* allele or the *Pinb-D1b* allele, no other *hardness* alleles were detected (Table 3). All winter wheat varieties possessed the *Pinb-2v3* variant, whereas the spring wheat varieties possessed either the *Pinb-2v2* or the *Pinb-2v3* variant in ratios ranging from about 1:3 to 6:4 (Table 3). Consequently, all varieties could be assigned one of only six *Pin-D1/Pinb-2* haplotypes. Within a subset of these varieties, we analyzed the relationship between *Puroindoline-D1* alleles and *Puroindoline b-2* variants and kernel texture. The same 247 Washington G&E wheat varieties used in the kernel texture ANOVA (Tables 1, 4, 5, 6, 7), and encompassing a total of 2,812 grain samples were analyzed. On the basis of *Pin-D1* and *Pin b-2* haplotypes, all 247 wheat varieties could be divided into six groups: *Pina-D1a/Pinb-D1a/Pinb-2v2*, *Pina-D1a/Pinb-D1a/Pinb-2v3*, *Pina-D1a/Pinb-D1b/Pinb-2v2*, *Pina-D1a/Pinb-D1b/Pinb-2v3*, *Pina-D1b/Pinb-D1a/Pinb-2v2* and *Pina-D1b/Pinb-D1a/Pinb-2v3*. Preliminary attempts at analyzing combined data sets across crop years were considered unreliable due to the highly unbalanced nature of the study and the influence of individual varieties (varieties changing across crop years, albeit balanced within crop year) (data not shown). Therefore, the kernel texture of wheat varieties was examined graphically (Figs. 1, 2, 3, 4) for each crop year by winter–spring, soft–hard combination for the six *Pin* haplotype groups described above. The largest group was comprised of the soft winter wheat varieties, but they were invariant for both the *Pin-D1* and *Pinb-2* loci (Table 3), and consequently no contrast was possible (overall mean SKCS Hardness Index = 31.4, n = 1,007 grain samples) (Fig. 1; Table 4). Among the soft spring wheat varieties, the average SKCS kernel texture for the *Pinb-2v2* varieties was 24.0 (n = 101) versus the *Pinb-2v3* haplotype with an average kernel texture of 26.1 (n = 400) (Fig. 2; Tables 3, 5). All of the hard winter wheat varieties possessed the *Pinb-2v3*

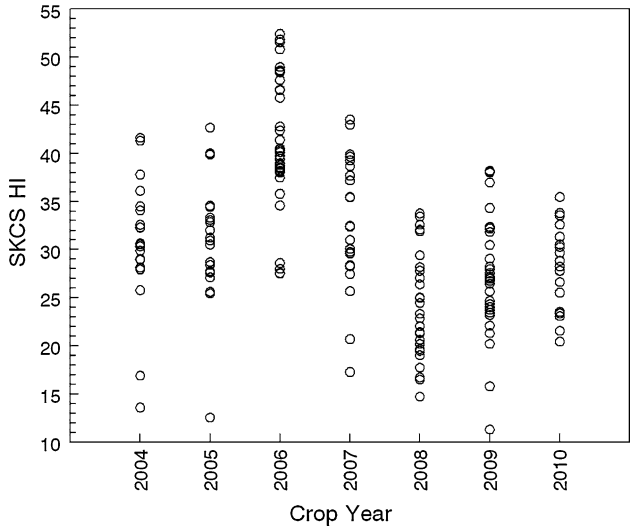

Fig. 1 Influence of *Puroindoline D1* and *Puroindoline b-2* variant alleles on kernel texture of soft white winter wheat varieties grown in 7 crop years. All varieties carry the *Pina-D1a/Pinb-D1a/Pinb-2v3* haplotype. Each *circle* represents the mean of the variety across locations (see Table 4)

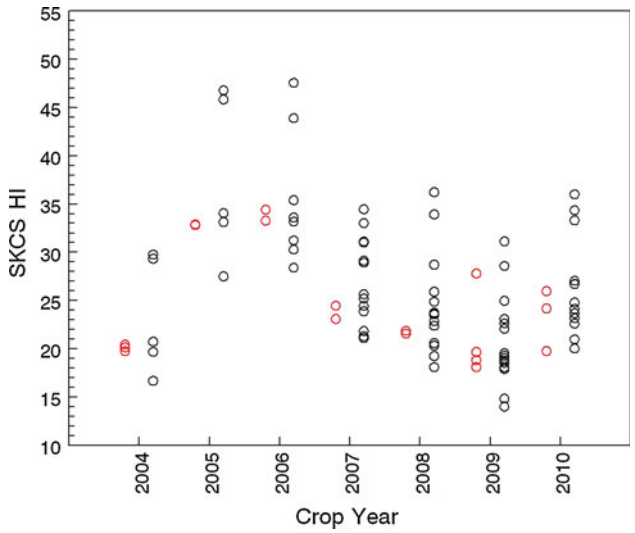

Fig. 2 Influence of *Puroindoline D1* and *Puroindoline b-2* variant alleles on kernel texture of soft white spring wheat varieties grown in 7 crop years. All varieties carry the *Pina-D1a/Pinb-D1a* soft haplotype and either the *Pinb-2v2* (*circles* offset to the *left* of each crop year) or *Pinb-2v3* (*circles* offset to the *right* of each crop year) haplotype. Each *circle* represents the mean of the variety across locations (see Table 5)

variant, but differed for *hardness* mutation: either the *Pina-D1b* or *Pinb-D1b* (Table 3). The average SKCS kernel texture was 77.8 for *Pina-D1b* varieties ($n = 57$) versus 71.5 for *Pinb-D1b* varieties ($n = 587$) (Fig. 3; Table 6). It should be noted that the cultivar Weston (HRW) was present in the nurseries in 2004 through 2007. Previous work (Morris and King 2002) showed that this cultivar is

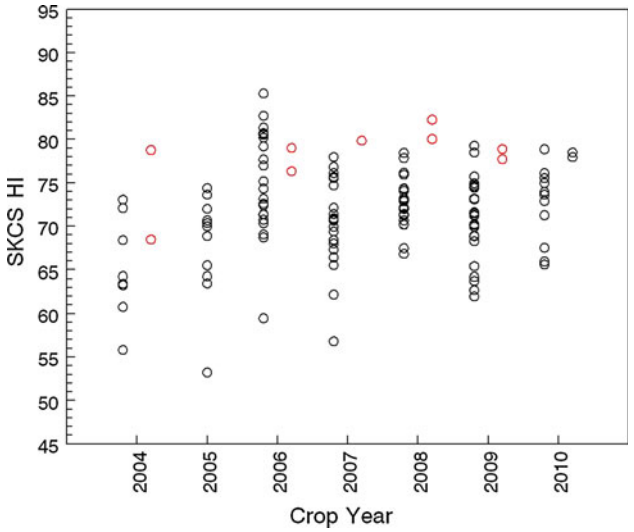

Fig. 3 Influence of *Puroindoline D1* and *Puroindoline b-2* variant alleles on kernel texture of hard winter wheat varieties grown in 7 crop years. All varieties carry the *Pinb-2v3* haplotype, and either the *Pina-D1b* (*circles* offset to the *right* of each crop year) or the *Pinb-D1b* (*circles* offset to the *left* of each crop year) hard mutation. Each *circle* represents the mean of the variety across locations (see Table 6)

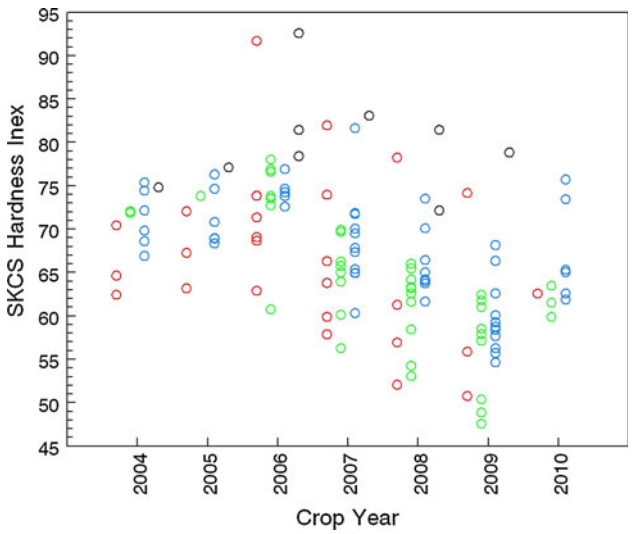

Fig. 4 Influence of *Puroindoline D1* and *Puroindoline b-2* variant alleles on kernel texture of hard spring wheat varieties grown in 7 crop years. Varieties carry either the *Pina-D1a/Pinb-D1b/Pinb-2v2* (*circles* offset furthest to the *right* of each crop year), the *Pina-D1a/Pinb-D1b/Pinb-2v3* (*circles* offset immediately to the *right* of each crop year), the *Pina-D1b/Pinb-D1a/Pinb-2v2* (*circles* offset immediately to the *left* of each crop year), or the *Pina-D1b/Pinb-D1a/Pinb-2v3* (*circles* offset furthest to the *right* of each crop year). Each *circle* represents the mean of the variety across locations (see Table 7)

actually a mixture of hard and soft kernel types. In all 4 crop years, Weston was notably the softest (Fig. 3). Among the hard spring wheat varieties, four haplotypes were

present (Table 3). Their respective mean SKCS kernel texture values were: *Pina-D1a/Pinb-D1b/Pinb-2v2*, 66.9 ($n = 135$); *Pina-D1a/Pinb-D1b/Pinb-2v3* 64.4 ($n = 210$); *Pina-D1b/Pinb-D1a/Pinb-2v2*, 67.4 ($n = 265$); and *Pina-D1b/Pinb-D1a/Pinb-2v3*, 79.0 ($n = 50$) (Fig. 4; Table 7). These results support previous reports that the *Pina-D1b* 'A-null' allele is harder than the *Pinb-D1b* allele, but do not indicate a prominent role for *Pinb-2* variants 2 and 3 in kernel texture.

Discussion

The *Puroindoline D1* genes have a dramatic impact on kernel texture, a significant effect on processing quality and may play a role in plant disease defense (Chen et al. 2007; Feiz et al. 2009; Giroux and Morris 1997, 1998; Lillemo and Morris 2000; Ma et al. 2009; Zhang et al. 2010, 2011). Recently, a new puroindoline gene, *Puroindoline b2*, was reported (Wilkinson et al. 2008). The role of the *Pinb-2* gene and in particular variants that appear allelic have not been adequately resolved. Since the related genes, *Pina-D1* and *Pinb-D1*, exert a prominent role in kernel texture variation, the *Pinb-2* genes should be examined in this context. Further, all the puroindoline genes appear to be highly conserved and as such, diagnostic markers of germplasm phylogeny.

It is now well established that all soft wheats possess the wild-type alleles, *Pina-D1a/Pinb-D1a* (Morris 2002; Bhave and Morris 2008a, b; Morris and Bhave 2008). Among hard wheats, studies have shown that *Pinb-D1b* was the predominant genotype compared with *Pina-D1b* (Lillemo and Morris 2000; Morris et al. 2001; Xia et al. 2005). In this study, we analyzed the frequency of *Pina-D1b* and *Pinb-D1b* among four hard wheat groups (hard red winter, hard white winter, hard red spring and hard white spring), and found that 94 of 101 hard winter wheats (red and white) carried *Pina-D1b* (Table 3). In contrast, *Pina-D1b* was the predominant genotype (56.4%) among hard red spring wheat varieties, whereas *Pinb-D1b* was more prevalent among the hard white spring wheats.

At the *Pinb-2* locus, variant 3 (here designated *Pinb-2v3*) was the predominant variant in all but the hard red spring group (from 60.0 to 100.0%; 84.5% overall) (Table 3). This result is in agreement with previous reports (Chen et al. 2010a, b, 2011; Wilkinson et al. 2008). In the hard red spring group, however, *Pinb-2v2* was the predominant genotype (58.2%). Our assumption is that these gene frequencies most likely reflect founder effects (germplasm introductions) and the flow of introduced germplasm to North America. In this regard, there was a distinct division between the winter and spring groups, and highlights a distinctive feature of U.S. Pacific Northwest wheat germplasm structure. Chen et al. (2010b, 2011) advanced that the preponderance of the *Pinb-2v3* variant was associated with superior grain yield traits compared to the *Pinb-2v2* allele in wheat.

The major classes of kernel texture are conditioned by the *Hardness* locus, which controls most of the variation in grain texture in wheat and results from the action of the closely linked genes puroindoline a and puroindoline b (Law et al. 1978; Mattern et al. 1973; Cane et al. 2004; Chen et al. 2006; Eagles et al. 2006; Giroux et al. 2000; Martin et al. 2001) (Tables 4, 5, 6, 7; Figs. 1, 2, 3, 4). It is still inconclusive whether *Puroindoline b2* variants affect kernel texture, even though past studies have indicated that the *Puroindoline b2* gene may exert some impact among soft wheat cultivars (Chen et al. 2010b, 2011). In this study, we analyzed the association of *Puroindoline D1* alleles and *Puroindoline b2* variants with kernel texture. Results show that unlike *Pin-D1* genotypes which have a pronounced association with kernel texture, a role for *Puroindoline b2* variants could not be ascertained. At most, it would appear that a putative role would be minor and less than other unidentified genetic contributions (Tables 4, 5, 6, 7; Figs. 1, 2, 3, 4). More carefully controlled genetic studies with segregating material, recombinant inbred lines, doubled haploids, etc., would be required. Biologically, the primary limitation to the role of the *Pin-b2* genes may be that they are expressed at possibly less than 10% the levels of transcripts encoding *Pina-D1* and *Pinb-D1* (Wilkinson et al. 2008).

Regardless of a possible role of *Pinb-2*, considerable intervarietal variation in kernel texture was identified. In fully balanced factorial multi-location trials, the variety range in SKCS Hardness Index was on the order of 15–25 (Tables 4, 5, 6, 7) against a range in LSDs of 2.8–5.6. These results clearly point to the presence of minor genes (Mattern et al. 1973; Pomeranz and Willams 1990; Sourdille et al. 1996; Perretant et al. 2000). Using a set of 187 doubled haploid lines derived from the cross between cvs. Courtot and Chinese Spring, Perretant et al. (2000) found minor kernel texture QTLs on chromosomes 1A and 6D, which explained 3 and 5.5%, respectively, of the phenotypic variance. These genes would be independent of the *Pin-b2* loci that appear on homoeologous group 7 chromosomes (Chen et al. 2007, 2011). Our analysis of genotypic variance (σ^2) (Tables 4, 5, 6, 7) indicated that a considerable portion of the observed total variance was related to varieties and as such, should provide a target for applied plant breeding. From our experience in wheat cultivar development, these differences in kernel texture among wheat varieties are of technological importance and should be pursued.

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