

The distal portion of the short arm of wheat (*Triticum aestivum* L.) chromosome 5D controls endosperm vitreosity and grain hardness

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Abstract Kernel vitreosity is an important trait of wheat grain, but its developmental control is not completely known. We developed back-cross seven (BC₇) near-isogenic lines in the soft white spring wheat cultivar Alpowa that lack the distal portion of chromosome 5D short arm. From the final back-cross, 46 BC₇F₂ plants were isolated. These plants exhibited a complete and perfect association between kernel vitreosity (i.e. vitreous, non-vitreous or mixed) and Single Kernel Characterization System (SKCS) hardness. Observed segregation of 10:28:7 fit a 1:2:1 Chi-square. BC₇F₂ plants classified as heterozygous for both SKCS hardness and kernel vitreosity ($n = 29$) were selected and a single vitreous and non-vitreous kernel were selected, and grown to maturity and subjected to SKCS analysis. The resultant phenotypic ratios were, from non-vitreous kernels, 23:6:0, and from vitreous kernels, 0:1:28, soft:heterozygous:hard, respectively. Three of these BC₇F₂ heterozygous plants were selected and 40 kernels each drawn at random, grown to maturity and subjected to SKCS analysis. Phenotypic segregation ratios were 7:27:6, 11:20:9, and 3:28:9, soft:heterozygous:hard. Chi-square

analysis supported a 1:2:1 segregation for one plant but not the other two, in which cases the two homozygous classes were under-represented. Twenty-two paired BC₇F₂:F₃ full sibs were compared for kernel hardness, weight, size, density and protein content. SKCS hardness index differed markedly, 29.4 for the lines with a complete 5DS, and 88.6 for the lines possessing the deletion. The soft non-vitreous kernels were on average significantly heavier, by nearly 20%, and were slightly larger. Density and protein contents were similar, however. The results provide strong genetic evidence that gene(s) on distal 5DS control not only kernel hardness but also the manner in which the endosperm develops, viz. whether it is vitreous or non-vitreous.

Introduction

Wheat (*Triticum aestivum* L.) is a major food crop worldwide. There are three significant factors that contribute to its prominence: it is broadly adapted and productive, it produces grain that is easily stored and transported, and the grain can be used in innumerable nutritious and appealing foods. Bread, noodles, cookies and cakes are major food types made from wheat. In most cases, the entire kernel (caryopsis) is not used, but rather the inner endosperm is separated from the outer bran coverings and reduced via the rolling milling process. Consequently, endosperm properties such as kernel texture, protein content and vitreosity are critical and practical considerations.

The molecular genetic basis for kernel texture in wheat is well established. Puroindoline a and puroindoline b function in an unknown manner to soften the endosperm. Both genes occur in close proximity (~18 kb) (Chantret et al. 2005) at the *Hardness* (*Ha*) locus on the distal end of

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the short arm of chromosome 5D (Law et al. 1978). When both puroindolines are in their “functional” state, endosperm texture is soft. Hard kernel texture in *T. aestivum* results from a mutation in either *Puroindoline a* or *Puroindoline b* (*Pina* and *Pinb*, respectively). Complementation studies by Beecher et al. (2002) and Martin et al. (2006) supported the role of puroindolines in controlling kernel softness. Durum wheat (*T. turgidum* ssp. *durum*) is notable in that it possesses neither *Pina* nor *Pinb*, and it has exceptionally hard kernels. Introduction of the puroindolines into durum resulted in soft kernels (Morris et al. 2011).

Vitreosity refers to the physical properties that affect the visual appearance of wheat grains (Morris 2010). Vitreous kernels have a continuous endosperm that refracts light in a manner such that the kernel appears ‘glassy’ (hence ‘vitreous’). In non-vitreous kernels (also called ‘yellow berry’ or ‘piebald’), the endosperm is discontinuous, containing minute airspaces. This structure refracts light differently than continuous vitreous endosperm such that the kernel appears “chalky.” Vitreosity is influenced by the environment, most notably by nitrogen fertility. Higher N fertility is associated with a higher proportion of vitreous kernels and a higher grain protein content. Under the US Official Standards for Grain, subclasses for Hard Red Spring wheat and Durum wheat are based on percentage vitreous kernels (consequently this grading criterion influences price and market segregation).

The prevailing understanding of wheat grain vitreosity versus “hardness” has been reviewed by Anjum and Walker (1991), wherein “hardness” is generally defined as the resistance of the kernel to deformation (i.e. crushing) (Greenaway 1969) with most variation associated with the 5DS *Ha* locus (Law et al. 1976). Anjum and Walker (1991) noted that both soft and hard wheats may produce vitreous or non-vitreous kernels. Dhaliwal et al. (1986) studied the inheritance of yellow berry among the progeny of six bi-parental crosses, and Chinese Spring monosomics × CIMMYT breeding line ‘WL711’. The bi-parental crosses indicated two or three dominant genes (Chi-square analysis on F₃ families). The monosomic analysis identified major dominant genes on chromosomes 1A and 7A; no association was reported with 5D. Nelson et al. (1995) mapped a QTL for kernel vitreosity to the distal portion of 5DS in the ‘Synthetic’ × ‘Opata 85’ recombinant inbred line (RIL) population (linkage to marker *Xfbb328*, 33% phenotypic variation; *Hardness* locus, sensu Law et al. (1976), was not determined). Weightman et al. (2008) studied a set of doubled haploids from cvs. Beaver × Soissons, and stated that, “there appeared to be no direct relationship between alleles at the *Ha* locus and vitreosity.”

The deletion stocks of Chinese Spring (CS) wheat, created by Endo and Gill (1996), have been exceptionally valuable for physically mapping traits and genes. Four

deletion lines involving some portion of chromosome 5D short arm were isolated (Endo and Gill 1996); the most distal is CS5DS del-2. CS5DS del-2 has an arm fraction length (FL) of 0.78 (<http://www.k-state.edu/wgrc/Germplasm/Deletions/group5.html>). Gill et al. (1996) and Tranquilli et al. (2002) physically mapped the *Grain Softness Protein-1* (*GSP-1*) gene to that portion of CS5DS deleted in CS5DS del-2. *GSP-1* is related to the puroindolines and resides at the Hardness locus (Morris 2002; Chantret et al. 2005; Bhave and Morris 2008a, b). Tranquilli et al. (2002) further showed that CS5DS del-2 also lacked the *Pina* and *Pinb* genes. CS5DS del-2 was significantly harder (Single Kernel Characterization System Hardness Index = 93.7) than the CS euploid parent (SKCS HI = 61.3), consistent with the complete loss of *Ha*, *Pina* and *Pinb*.

We have developed a number of near-isogenic lines in wheat using the soft white spring wheat cv. Alpowa as the recurrent parent (Morris and King 2008). As this work continues, we introgressed as donor parent CS5DS del-2 as a member of this series. In this report, we describe the perfect association among BC₇F₂ and BC₇F₃ segregants for kernel hardness, vitreosity and the presence/absence of the distal portion of CS5DS. The results provide new information as to the regulation of wheat grain development and utilization.

Materials and methods

BC₇F₂ and BC₇F₃ plants with the pedigree Alpowa/2/ Chinese Spring 5DS del-2/Alpowa/6*Alpowa were grown in a glasshouse at the Washington State Univ. Plant Growth Facility in 3.0-L plastic pots and artificial growth media (a peat based SunShine mix product LC1, Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia). The greenhouse environment was set at 21–24 °C day, 15–18 °C night. A 16-h photoperiod was used and supplemented with 350 μEinstein of light using 1,000-W high pressure sodium lamps. Nutrients were provided weekly in irrigation water (a peat lite special 20–10–20, N–P–K, mixture delivered at 100 ppm N). Plants were harvested at maturity, threshed and kernels subjected to Single Kernel Characterization System 4100 (SKCS) (Perten Instruments, Springfield, IL) analysis on a per plant basis (Fig. 1). The SKCS weighs and crushes individual kernels and converts the force-crush profile to a unit-less Hardness Index (HI). Kernel weight is expressed on an as-is moisture basis. The approximate moisture content of the kernels was 7.5–8.8%, which is the equilibrium moisture content of the laboratory environment.

An analysis of vitreous/non-vitreous kernel phenotypes versus SKCS kernel texture was evaluated in 46 BC₇F₂

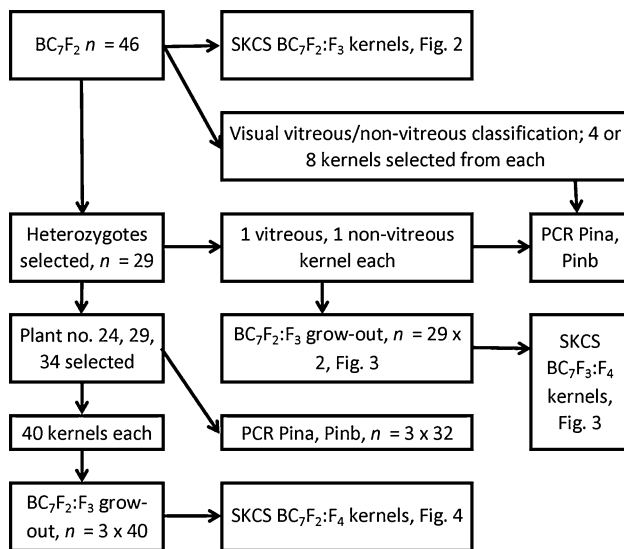


Fig. 1 Schematic representation of generations, selections and analysis of BC₇ wheat germplasm possessing or lacking the distal portion of chromosome 5DS. All plants were in the recurrent parent background of Alpowa soft white spring wheat cultivar. The distal deletion of 5DS was derived from Chinese Spring 5DS del-2

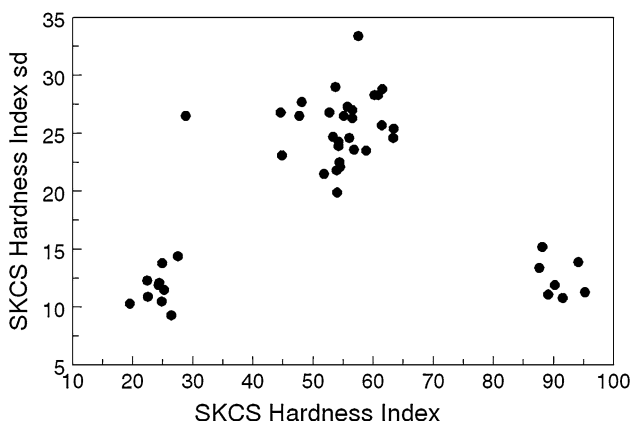


Fig. 2 Plot of SKCS kernel Hardness Index versus Hardness Index standard deviation of BC₇F₂ wheat plants segregating for a complete 5DS chromosome possessing the *Hardness* locus and puroindoline a and b genes derived from Alpowa soft white spring cultivar, or a distal deletion of 5DS derived from Chinese Spring 5DS del-2. BC₇F₃ kernels were crushed

plants (50 kernels each) (Figs. 1, 2). Subsequently, from each of the 29 individual BC₇F₂ plants classified as heterozygotes (plant ‘no. 19’ with a ‘soft’ HI but a high HI SD, 28.8 ± 26.5 , was included), a single vitreous and a single non-vitreous kernel were visually selected (outer appearance of the kernel) by Gail Jacobson. These plants were grown to maturity in a glasshouse, and the BC₇F₃:F₄ kernels were subjected to SKCS analysis (40 kernels each) (Fig. 1). These 58 plants were included in tissue sampling for DNA (see below). An additional four vitreous and four non-vitreous kernels were visually selected from each of

these 29 BC₇F₂ heterozygous plants by the senior author (outer appearance of the kernel) (Fig. 1). In both samplings, unambiguous phenotypes (clearly vitreous or non-vitreous) were purposefully selected. In addition, four kernels were drawn at random from each of the plants from the soft and hard classes (i.e. classified as homozygous for the presence of a complete 5DS chromosome or lacking the distal 5DS portion) (Fig. 1). The kernels of these homozygous plants were uniform for vitreous/non-vitreous phenotype. All of these selected kernels were grown to the 3–4 leaf stage and were included in tissue sampling for DNA (see below). To augment the above-sampling schemes, three heterozygous BC₇F₂ plants were selected on the following basis: the ‘softest’ of the heterozygous classification group (‘no. 24’, SKCS HI 44.6 ± 26.8), the ‘hardest’ (‘no. 34’, HI 63.4 ± 25.4), and one plant near the middle of the range and with the highest SD (‘no. 29’, HI 53.7 ± 29.0). From these three plants, 40 BC₇F₃ kernels were drawn at random from each. These plants were grown to maturity and subjected to SKCS (40 kernels each). In total, 450 BC₇F₃ seedlings (3–4 leaf stage) were tissue harvested for DNA and PCR (see below). In summary, this sampling included 1 + 1 and 4 + 4 kernels (vitreous + non-vitreous) from the 29 heterozygotes, 4 kernels from each of the 10 homozygotes, and 40 kernels from each of the 3 selected heterozygotes (Fig. 1).

Kernel density was measured using an air pycnometer (MultiPycnometer model MVP-1, Quantachrome Corp., Syosset, NY, USA) using the “small” sample cup (ca. 14 g capacity) and compressed air following the manufacturer’s instructions. Five BC₇F₃ soft and hard full-sib plant pairs (10 total) (plant nos. 3, 5, 8, 18, 22) were selected at random and used in the analysis. Six replicate readings were recorded for each plant. Kernels from these same plants were used for single-kernel protein determinations.

Protein ($N \times 5.7$) was determined by the Dumas combustion method (AACC International 2011) (Approved Method 46-30) (model FP-528, Leco Corp., St. Joseph, MI, USA), and expressed on an as-is moisture basis. Six replicate kernels from each soft and hard full-sib pair were lightly crushed with a hammer; the kernel was held inside a folded weighing paper. The crushed kernel was transferred to a tared foil wrapper (Tin Foil Cup, Part no. 502-186, Leco Corp.).

Field emission scanning electron microscopy was conducted using a Quanta 200F (FEI Co., Hillsboro, OR). Two BC₇F₂:F₃ full sibs (‘no. 29-3’ non-vitreous, SKCS HI 28 ± 13 , and ‘no. 29-25’ vitreous, SKCS HI 89 ± 15) were selected for imaging. Wheat kernels (BC₇F₄) were cleaved with a razor blade perpendicular to the long (germ-to-brush) axis, taking care to produce a “fracture” break as opposed to a cutting of the tissue. Samples were mounted on aluminum microscopy specimen stubs with the exposed

endosperm facing upward, and gold sputtered for 8 min to 200 Å (Technics Hummer V, Anatech, San Jose, CA, USA). Preliminary examinations were conducted at up to 2,600× magnification. Magnifications of approximately 30 and 600× were selected. The exposed endosperm “landscape” of each grain was surveyed at various locations. Central endosperm was selected, avoiding close proximity to the outer bran or the central pigment strand.

Genomic DNA was isolated by the method of Riede and Anderson (1996) from leaf tissue collected from individual seedlings. PCR for puroindoline a and puroindoline b gene coding regions were conducted as follows using an MJ Research PTC-200 Thermo Cycler (Watertown, MA, now part of BioRad, Hercules, CA, USA). The temperature regimen consisted of a 3-min initial denaturation step at 95°C, followed by 45 cycles of: (1) 95°C for 50 s, (2) 30 s at the annealing temperature (see below), and (3) a 60 s extension step at 72°C. Reaction conditions consisted of 1× PCR buffer (New England Biolabs, Ipswich, MA), 0.4 pmol/μL of each primer, 0.2 mM each dNTP, 0.02 U/μL *Taq* polymerase (5 Prime, Gaithersburg, MD) and approximately 50 ng genomic DNA in a 25-μL reaction volume. The primers pinaff2 (5′agtacacggaacatcacatctcaaa3′) and pinafla3 (5′accactktattgtcacacgcag3′) were used at an annealing temperature of 63°C to amplify a 699 bp product containing the Puroindoline a gene. The primers pinbff12 (5′attctctgttgctcgcaag3′) and pinbflr3 (5′ctccaacaacatcaatatacaagggtg3′) were used to amplify a 745 bp product containing the Puroindoline b gene using an annealing temperature of 55°C. PCR products were sequenced using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Waltham, MA) using the primers pinaflseqf1 (5′caccagtttgtgtgct3′) and pinbflseqf1 (5′ttagtataccaacttaattg3′) for *Puroindoline a* and *Puroindoline b*, respectively. The sequencing reactions were precipitated with ethanol and EDTA and analyzed on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA). Sequence reads were analyzed using the programs Finch TV and Biology Workbench 3.2 (<http://workbench.sdsc.edu>).

The *PPO-D2b* gene is located on chromosome 2A of Alpowa and was chosen as a positive DNA control. The primers D2bNtF1a (5′accaccgcccccgca3′) and D2binR1a (5′acgacctgttgctgtagata3′) were used to amplify an 873 bp fragment of *PPO-D2b* under conditions identical to that described for the puroindoline a gene above, with the sole exception that the reaction mixture contained 1 M Betaine (Sigma-Aldrich, St. Louis, MO, USA).

Chi-square analysis was conducted using PROC FREQ in the statistical package SAS v9.1 (SAS Institute, Raleigh, NC, USA). ANOVA was conducted using PROC GLM in SAS.

Results

SKCS Hardness Index (HI) results on the 46 BC₇F₂ plants (BC₇F₃ kernels crushed) are presented graphically in Fig. 2. Phenotypically, the plants, with one exception (no. 19, see above), assorted themselves into three distinct groupings. Ten plants had SKCS HI values from 19.5 to 27.5, and SKCS HI SD from 9.3 to 14.4. Based on these results, these ten plants were classified as homozygous soft and likely possessed a complete 5DS chromosome, including the *Hardness* locus and the *Pina-D1a/Pinb-D1a* genes from Alpowa (i.e., they “lacked” the deletion). Twenty-eight plants had SKCS HI values from 44.6 to 63.4, and SKCS HI SD from 19.9 to 33.4. These plants were classified as heterozygous, wherein the individual seeds within the spike had segregated for the presence/absence of a complete 5D short arm (and the *Hardness* locus). Seven plants had SKCS HI values from 87.6 to 95.2, and SKCS HI SD from 10.8 to 15.2. These plants were classified as homozygous for the 5DS deletion (very hard kernels and lacking the *Hardness* locus and puroindolines). Their kernel texture was similar to that observed for durum wheat (Morris et al. 2011). One plant (no. 19) could not be unambiguously classified as it had an SKCS HI of 28.8, which would have placed it in the soft class, but its SKCS HI SD was 26.5, suggestive of a heterozygote. Ignoring this one plant, the observed segregation was 10:28:7. Chi-square analysis returned a value of 3.09 with a *P* value of 0.21, indicating that the data fit a 1:2:1 segregation.

In concert with the SKCS kernel texture results, the group of plants classified as soft and therefore possessing a complete 5DS chromosome possessed uniformly non-vitreous kernels. Conversely, the group with high SKCS HI (>87) and classified as lacking the terminal portion of the 5DS chromosome was uniformly vitreous. Those plants classified as heterozygotes exhibited a range of kernels from non-vitreous to vitreous and some that were intermediate which were difficult to classify by eye (data not shown).

The results of the first part of the BC₇F₂:F₃ grow-out are presented in Fig. 3. These data are the SKCS hardness of the 58 BC₇F₂:F₃ plants derived from the single vitreous and non-vitreous kernel selections drawn from each of the 29 heterozygous BC₇F₂ plants. SKCS analysis was on BC₇F₃:F₄ kernels. Clearly, visual selection based on the kernel vitreous/non-vitreous phenotype was highly associated with that plant’s *Hardness* genotype. When selecting a vitreous kernel, only once was a heterozygote selected. When selecting non-vitreous kernels, six times out of 29 a heterozygote was selected. In no case was a homozygous plant of the ‘wrong’ genotype observed (i.e. a non-vitreous

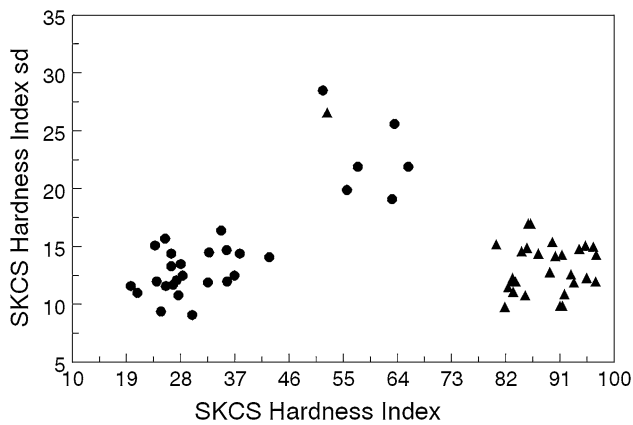


Fig. 3 Plot of SKCS kernel Hardness Index versus Hardness Index standard deviation of BC₇F₂:F₃ wheat plants. Heterozygous BC₇F₂ plants ($n = 29$) were selected based on the results shown in Fig. 1. From each plant, a single vitreous (triangles) or non-vitreous (dots) kernel was visually selected, grown to maturity and its BC₇F₄ kernels were crushed. Plants were segregating for a complete 5DS chromosome possessing the *Hardness* locus and puroindoline *a* and *b* genes derived from Alpowa soft white spring cultivar, or a distal deletion of 5DS derived from CS 5DS del-2

kernel never produced a ‘hard’ plant, and a vitreous kernel never produced a ‘soft’ plant).

PCR was used to evaluate the *Puroindoline a* genotype of these 58 BC₇F₃ plants (Fig. 1). Of the vitreous and non-vitreous kernels selected from the 29 heterozygous BC₇F₂ plants, all of the 23 homozygous soft plants (non-vitreous) produced positive *Pina* PCR product. Of the non-vitreous kernel-derived heterozygotes, 5 of 6 were *Pina* positive. None of the 28 hard plants nor any of the single vitreous kernel-derived heterozygous plants produced a *Pina* product. All of these DNA samples, however, produced a *PPO-D2b* product in PCR (positive control) (data not shown).

A second set of 4 vitreous and 4 non-vitreous kernels was independently and visually selected from each of 29 BC₇F₂ plants classified as heterozygous for a total of 232 kernels (Figs. 1, 2). Each was analyzed for *Puroindoline a* and *b* via PCR as markers for the 5DS deletion. Of the 116 kernels selected by eye as being non-vitreous, 100 were *Pina* or *Pinb* positive. Conversely, of the 116 vitreous kernels, 21 were positive for *Pina* or *Pinb* and 95 produced no product for either gene. Since this larger sampling of kernels was not grown out, their SKCS kernel texture phenotype was not determined. However, based on the 2-kernel sampling (Fig. 3), an 88% correct phenotypic selection rate could be expected. The 4 × 2 kernel sampling results were consistent, having an 84% correspondence between vitreous/non-vitreous kernel phenotype and PCR results. These estimates are likely low due to less than 100% successful PCR. For comparison, of the 40 kernels (4 from each of 10 plants) drawn from the ‘soft’ classified BC₇F₂ plants, 37 were positive for one or both *Pin* genes.

None of the 16 kernels (4 each plant) from 4 of the 7 ‘hard’ classified plants drawn at random produced any *Pin* positive results. These PCR results on the homozygous soft plants indicate that the PCR, in this case, was only about 92% efficient.

The second part of the BC₇F₂:F₃ grow-out examined the phenotypic segregation of unselected kernels (Fig. 1). Forty plants each of the previous three selected BC₇F₂ heterozygotes (plant nos. 24, 29, 34) were grown as BC₇F₃, and their BC₇F₄ kernels subjected to SKCS (Fig. 4). The phenotypic segregation ratios were 7:27:6, 11:20:9, 3:28:9, soft:heterozygous:hard for plants nos. 24, 29 and 34, respectively. Chi-square analysis provided strong support for only plant no. 29 conforming to an expected 1:2:1 segregation ($P = 0.90$). For plants nos. 24 and 34, the Chi-square values of 4.95 and 8.20 were associated with P values of 0.084 and 0.017, respectively. In both cases, the homozygous classes were under-represented. Whether the cause of this distortion was either genetically based or the result of sampling error could not be ascertained.

PCR was conducted on leaf tissue of 32 randomly selected progeny plants from each of these three selected BC₇F₂ heterozygotes. The *Pin* positive (one or both genes) versus *Pin* negative (neither gene) ratios were 20:12, 26:6 and 27:5, for plant nos. 24, 29 and 34, respectively. Chi-square analysis supported a 3:1 segregation for all three plant nos. (P values 0.10, 0.41, and 0.22, respectively).

ANOVA was used to test the effect of the 5DS deletion on kernel weight and size. The SKCS both weighs and measures the outer diameter (“size”) of each kernel before crushing it. Of the 29 BC₇F₂ heterozygous plants, 22

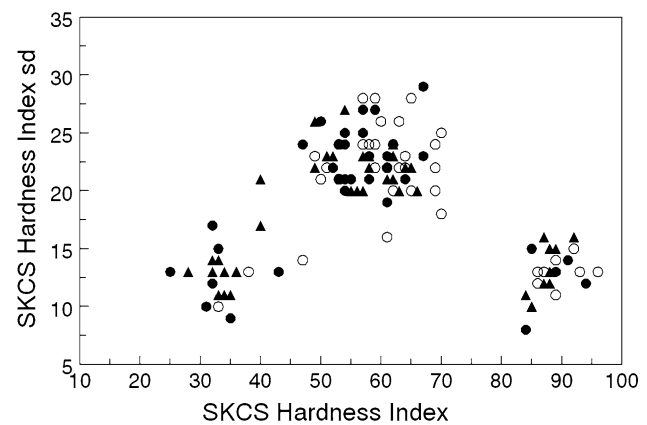
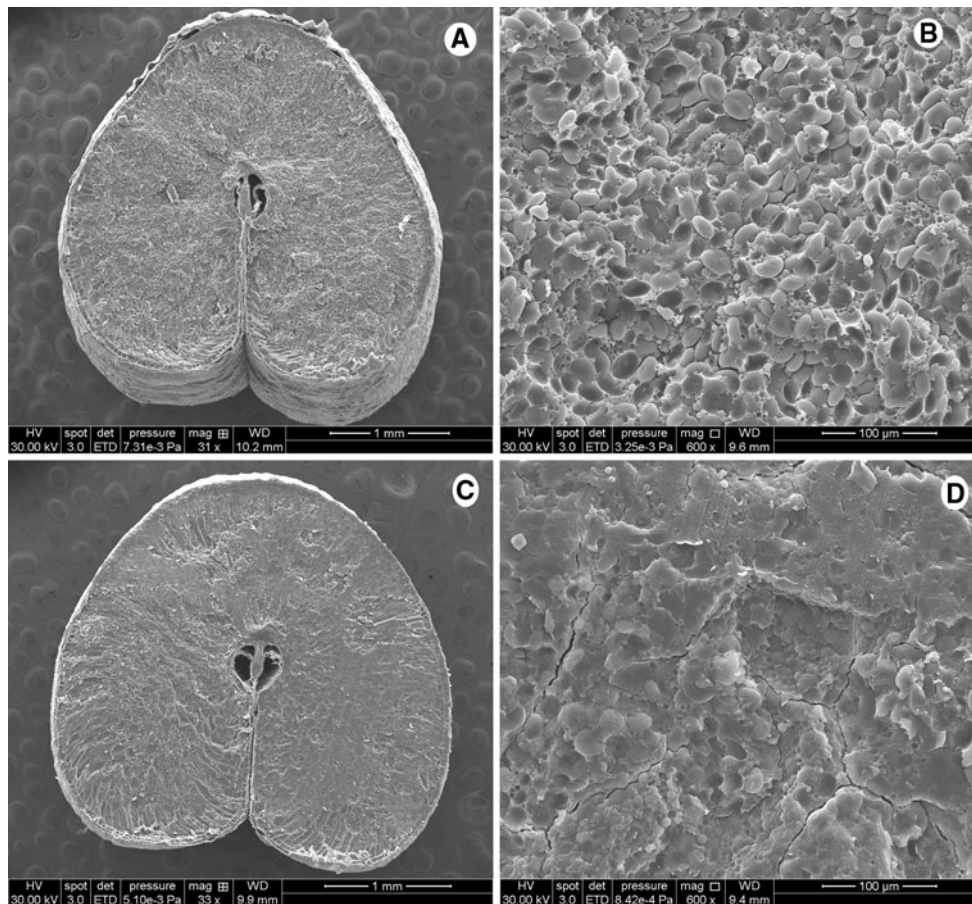


Fig. 4 Plot of SKCS kernel Hardness Index versus Hardness Index standard deviation of BC₇F₂:F₃ wheat plants derived from three heterozygous BC₇F₂ plants (nos. 24, 29, 34). From each plant, 40 kernels were drawn at random. BC₇F₄ kernels were crushed. Plants were segregating for a complete 5DS chromosome possessing the *Hardness* locus and puroindoline *a* and *b* genes derived from Alpowa soft white spring cultivar, or a distal deletion of 5DS derived from Chinese Spring 5DS del-2. Solid circles plant no. 24, filled triangles plant no. 29, and open circles plant no. 34

Table 1 Kernel texture, weight and size of 22 pairs, and density and protein content of five pairs of BC₇F₃ full-sib wheat plants with or without a distal deletion of chromosome 5DS

Chromosome 5DS status	Kernel texture (Hardness Index)	Kernel weight (mg)	Kernel size (mm)	Density (g cm ⁻³)	Protein (%)
Euploid	29.4	43.9	3.15	1.406	16.90
Deletion	88.6	36.9	3.06	1.412	16.75
LSD	4.1	1.6	0.06	–	–
<i>P</i> value	<0.0001	<0.0001	0.004	ns	ns

**Fig. 5** Field emission scanning electron micrographs of BC₇F₄ kernels derived from two BC₇F₃ full-sib plants. **a, b** A soft wheat kernel fractured transversely showing the non-vitreous kernel morphology and high degree of free starch granules (plant no. 29-3). **c, d** A hard wheat kernel fractured transversely showing the vitreous

kernel morphology and continuous endosperm matrix with no free starch granules (plant no. 29-25). The soft wheat has a complete 5DS chromosome, the hard wheat is lacking the distal portion of 5DS containing the *Hardness* locus and puroindolines. Scale bars are as shown

produced BC₇F₃ full sibs that included both a homozygous soft/non-vitreous and a homozygous hard/vitreous plant. The results of these paired sibs are presented in Table 1. As noted before, SKCS hardness differed markedly, 29.4 for the lines with a complete 5DS, and 88.6 for the lines possessing the deletion. The soft kernels were on average significantly heavier, by nearly 20%, than hard kernels and were slightly larger (diameters 3.15 vs. 3.06 mm). Density measurements on five soft and hard full-sib pairs indicated

similar densities (1.41 g cm⁻³). ANOVA of kernel protein returned a significant whole model ($F = 5.86$, $P < 0.0001$), but with a relatively modest model R^2 (0.51). ‘Plant’ and the ‘plant × vitreous state’ interaction terms were both significant, but not ‘vitreous state’ alone (data not shown) (Table 1).

Field emission scanning electron microscopy showed the dramatic difference in morphology and structure of the endosperm of the soft and hard kernel types (Fig. 5).

To our knowledge, this is the first ever image to document the effect of the wild-type *Pina* and *Pinb* genes compared to the complete absence of the *Ha* locus in BC₇F₃ full-sib BC₇F₄ kernels. Transverse cleavage of the kernels revealed the high degree of free starch granules in the non-vitreous kernel morphology (plant no. 29-3), and the continuous endosperm matrix with no free starch granules in the vitreous kernel (plant no. 29-25).

Discussion

Endosperm texture is a leading trait influencing the quality and utilization of wheat grain. The major classes of endosperm texture, namely soft, hard and durum, can be explained by the presence, absence or gene sequence of puroindoline a and b (Bhave and Morris 2008a, b; Morris 2002), which reside at the *Hardness* locus on the short arm of chromosome 5D (Chantret et al. 2005; Law et al. 1976, 1978; Martin et al. 2006). In the present report, we found the expected association between kernel texture phenotype, that is ‘soft’ and ‘durum hard’ and the presence/absence of the distal portion of 5DS (Figs. 2, 3, 4; Table 1). In this regard, the “removal” of the puroindolines/*Hardness* locus via the CS deletion stock of Endo and Gill (1996) is in a way the reverse of the introduction of the puroindolines/*Hardness* locus into durum (Morris et al. 2011). The complementation studies of Beecher et al. (2002) and Martin et al. (2006) show a clear requirement for both puroindoline proteins for soft endosperm. ‘Hardness’ mutations resulting from individual SNPs and a large deletion in *Pina* do not increase endosperm hardness in Alpowa to the level observed here in the back-cross seven 5DS deletion Alpowa NIL (Morris and King 2008; Feiz et al. 2009).

The striking feature of the present work was the observation of the apparently “perfect” and complete association between kernel vitreosity and the absence of the puroindolines/*Hardness* locus. This observation may not be a direct result of the puroindoline proteins, as no doubt other gene(s) are located on the portion of 5DS that was deleted. However, the results indicate a profound influence of a relatively small portion of the wheat genome on the growth and development of the endosperm (Fig. 5). This evocative result deserves further study.

Of further interest was the markedly heavier average kernel weight of the non-vitreous sibs among 22 paired BC₇F₃ non-vitreous and vitreous full sibs (Table 1). This result, too, deserves additional work. The heavier kernel weight (~20%) was only partially reflected in a larger kernel diameter (Table 1). Of some surprise, the density of non-vitreous and vitreous kernels was similar, as was protein contents (Table 1). It is a long-held view that

greater vitreosity is associated with higher protein content (Anjum and Walker 1991; Dhaliwal et al. 1986; Morris 2010; Weightman et al. 2008). Indeed, the official grain grading system in the US has institutionalized the importance and use of kernel vitreosity.

In closing, the present work reflects a convergence of several factors: the development of deletion stocks in wheat (Endo and Gill 1996), the creation of advanced back-cross derived near-isogenic lines (Morris and King 2008), the efficient “phenotyping” performed by the SKCS (AACC International 2011), and an appreciation for and knowledge of wheat grain texture and genetics (Bhave and Morris 2008a, b; Morris 2002).

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