

INCREASING WHEAT *HARDNESS* LOCUS FUNCTIONALITY BY
INCREASING *PURINDOLINE* COPY NUMBER
AND INTRODUCTION OF NOVEL ALLELES

by

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ABSTRACT

Wheat (*Triticum aestivum* L.) grain hardness affects many end product quality traits and is controlled primarily by the *Hardness* (*Ha*) locus that contains the *Puroindoline a* and *b* genes. All soft hexaploid wheats carry the same wild-type alleles *Pina-D1a* and *Pinb-D1a* and hard wheats carry a mutation in *Pina* or *Pinb*. In order to explore variation in the *Puroindolines* two approaches were pursued to increase *Ha* locus functionality. First *Ha* locus functionality was modified through a non-transgenic increase in *Pin* dosage. Here we tested the heritability and end product quality effects of increased *Pin* dosage in soft wheat. Previous experiments have suggested that grain softness can be enhanced by increasing *Ha* locus dosage via chromosome substitutions. Segregation data from a cross between a ‘Chinese Spring’ substitution lines with six doses of the *Ha* locus to the soft wheat variety ‘Vanna’ indicate that the substituted B genome *Ha* locus was not transmitted and that the A genome *Ha* locus was transmitted. Genotypes with the added *Pins* on the A genome produced seeds that were 7.4 hardness units softer. These softer double *Ha* genotypes were lower in flour yields, but produced flour with lower ash content, reduced starch damage, and smaller mean particle size. The second route to increased *Ha* functionality was via novel *Pin* alleles present in synthetic hexaploid wheat (*Triticum tauschii* L.). Previous research has indicated that the seeds of synthetic hexaploid lines containing the PINAc or PINBh proteins are significantly softer than those carrying other alleles. Here we show lines containing the PINAc / PINBh or PINAa / PINBj *Ha* locus exhibited increases in flour ash and flour particle size as well as decreased kernel weight relative to lines carrying the PINAa / PINBa *Ha* locus. Further lines containing the PINAa / PINBj *Ha* locus exhibited increases in grain hardness and flour yield as well as decreased protein content relative to lines carrying the PINAa / PINBa *Ha* locus. The resultant phenotypes revealed a potentially useful intermediate hardness with improved product quality properties. Increasing the range of novel *Ha* loci may lead to increased soft wheat quality and marketability.

CHAPTER 1
EFFECTS ON SOFT WHEAT (*TRITICUM AESTIVUM* L.) QUALITY OF
INCREASED PUROINDOLINE DOSAGE

Introduction

Wheat (*Triticum aestivum* L.) is an economically important crop in the United States with an approximate yearly production value of 7.19 billion dollars (USDA, 2004). Kernel texture is of paramount importance in the end product quality of commercial wheat varieties, determining the type of products that can be produced from the grain. Common hexaploid wheat varieties are categorized as “hard” or “soft” based on kernel texture. Kernel texture is associated with different end product properties resulting in hard wheat being best suited for breads and soft wheat being best suited for pastries (Reviewed in Morris and Rose 1996). In general terms, wheat quality is defined based on milling and baking quality. Kernel texture affects milling yield by influencing relative amounts of bran and flour recovered as well as affecting baking quality by influencing flour water absorption. Relative to hard wheats, soft wheats have reduced flour yield and yield flours with lower water retention capacities, smaller average particle size and less starch damage (Symes 1969; Rogers et al. 1993). Because of the relationship between starch damage and flour water absorption, soft wheat end product quality can be predicted using flour solvent retention capacities. Solvent retention capacity tests are used as an assay of flour characteristics and in predictive models for end product quality (Gaines 2004; Guttieri et al. 2001). In particular, sucrose retention capacity is directly

associated with sugar-snap cookie diameter (Gaines 2004) whereas sodium carbonate (Na_2CO_3) retention capacity is directly associated with damaged starch content (Guttieri et al. 2001). A specific baking test designed to measure the baking quality of soft wheat flours is the sugar-snap cookie spread assay as high quality soft wheats give flours with low starch damage that results in greater cookie diameter (Rogers et al. 1993).

Variation in wheat grain texture is controlled primarily by the *Hardness* locus (*Ha*), which is inherited simply (Symes 1964) and is located on the distal end of the short arm of chromosome 5D (5DS) (Mattern et al. 1973). The *Ha* locus contains the *Puroindoline a* (*Pina*), *Puroindoline b* (*Pinb*), and *Grain Softness Protein* (*Gsp*) genes (Sourdille et al. 1996; Giroux and Morris 1998; Jolly et al. 1993). The fact that *Ha* is a single locus in wheat is unique in the sense that common bread wheat, *Triticum aestivum*, is an allohexaploid ($2n = 6x = 42$) having three genomes designated as A, B and D. The creation of hexaploid wheat involved an initial divergence of the A, B and D genome diploid progenitor species followed by the hybridization of *Triticum urartu* (AA) and *Aegilops searsii* (BB) (McFadden and Sears 1946; Chantret et al. 2005). The resultant tetraploid durum wheat, *T. turgidum* (AABB), subsequently lost function of all *Ha* loci (Gautier et al. 2000). A more recent hybridization event combined *T. turgidum* with *Aegilops tauschii* (DD) producing modern *T. aestivum* hexaploid wheat (AABBDD) (McFadden and Sears 1946). In the commercially important hexaploid *T. aestivum* L. *Pins* are only expressed from the D genome; however there are transcriptionally active *Gsp* genes in the A, B and D genomes of hexaploid wheat (Gautier et al. 2000; Chantret et al. 2005).

While *Gsp* has not been functionally linked to grain hardness differences in wheat, both *Pina* and *Pinb* have been. Mutations in either *Pina* or *Pinb* have been found in all hard wheats examined (Giroux and Morris 1997, 1998; Morris 2002). Mutations in either of the *Pin* genes results in low levels of both proteins, PINA and PINB, associated with the surface of starch granules (Giroux and Morris 1997, 1998). PINA and PINB when associated with starch are collectively referred to as friabilin. Friabilin was originally defined as a 15kDa lipid-binding protein found on the surface of water-washed starch granules (Greenwell and Schofield 1986). The occurrence of friabilin on water-washed starch granules is a distinguishing characteristic between hard and soft wheats as friabilin is found at lower levels on hard wheat starch compared to soft wheat starch and is entirely absent from durum wheat (Greenwell and Schofield 1986). Demonstration that mutations in the *Pins* are causative to a hard phenotype has come from transgenic complementation studies in which soft phenotypes have been achieved in studies where hard *Pin* alleles were complemented with the wild-type *Pin* alleles (Beecher et al. 2002; Martin et al. 2006). Transgenic manipulations have also demonstrated that while both PINA and PINB levels limit grain softness in soft wheats, PINB is a greater limiting factor than PINA (Swan et al. 2006). Further, Hogg et al. (2005) demonstrated that transgenic overexpression of wild-type soft alleles in the hard elite background ‘Hi-Line’ had a significant effect on milling and baking traits. The research concluded that there was a significant correlation between expression of *Pins* and flour yield, break flour yield and flour ash content (Hogg et al. 2005). Non-transgenic manipulations of the puroindolines in hexaploid wheats are limited due to limited *Pin* allelic variation. In fact,

only eight *Pin* mutations have been observed thus far, all but two of which are in *Pinb* (Massa et al. 2004) and all soft wheats carry the *Pina-D1a* and *Pinb-D1a* alleles. Diploid wheats, however, contain a substantial number of polymorphisms in both *Pina* and *Pinb* that could be introduced into the hexaploid genome (Gedye et al. 2004) by either replacing the native *Pin* genes or by increasing expression levels of native *Pins*.

The majority of grain hardness variation observed between hard and soft wheats is associated with sequence variation in the *Puroindolines* (Giroux and Morris 1997, 1998). Kernel texture variation between soft wheats is presumably controlled by other factors such as protein content as well as the interaction of minor genes (Reviewed by Anjum and Walker 1991). Increasing grain softness in soft hexaploid wheats using the *Puroindolines* may be possible via crosses with wheat chromosomal substitution lines to increase the functional dose of *Ha*.

Wheat chromosomal substitution series have been used in the past to determine gene locations (Morris et al. 1966) and to introgress genetic material into desired backgrounds. Chromosome substitution lines in which inactive or absent A and B genome *Ha* loci in hexaploid wheats were replaced with active forms of diploid *Ha* were created by See et al. (2004). They demonstrated that the addition of diploid A and B genome *Ha* imparted a decrease in grain hardness in hexaploid wheat. The introgressions were done in the variety 'Chinese Spring' (CS) which is an oddity in the sense that it is genetically soft, but phenotypically hard having a Single Kernel Characterization System (SKCS) value more typical of a hard variety. In the CS background, for each additional introgressed A or B genome *Ha*, hardness decreased by approximately 10 SKCS hardness

units (See et al. 2004). See and coworkers (2004), however, did not demonstrate if the substituted loci could be stably transmitted in the hexaploid genome in crosses to adapted wheat varieties or whether the grain hardness reductions would have measurable impacts upon soft wheat quality.

The objective of this study was to determine the heritability, functionality and end product quality effects of additional substituted copies of the *Ha* locus. The question of heritability was addressed by analyzing recombinant lines created by crossing a ‘Chinese Spring’ substitution line from See et al. (2004) that had three *Ha* loci with the Montana adapted soft wheat cultivar ‘Vanna’. Functionality was addressed through grain hardness and end product quality assays.

It is expected that the A genome *Ha* locus will be stably inherited (Kota and Dvorak 1985; Morris et al. 1966) while the B genome *Ha* locus may not be due to the documented variability of the B genome within *T. aestivum* (Peng et al. 2003). The increased *Ha* dosage is expected to impart significant, observable positive changes in soft wheat quality (Hogg et al. 2004). This study complements established literature on the impact of *Pin* overexpression on end product quality as well as supports a non-transgenic method of producing genetically stable, viable wheat genotypes with decreased grain hardness and enhanced soft wheat end product quality.

Materials and Methods

Genetic Material

The substitution line ‘Chinese Spring’ (CS) #12 was chosen from the set of lines produced by See et al. (2004) as it contained substituted *Ha* loci on both the A and B genomes (AmAmBsBsDD) of CS. This line was created by See et al. (2004) using chromosome substitution line TA6642 DS5A^m (5A) to introgress the functional A genome *Ha* and chromosome substitution line TA6562 DS5S^s (5B) to introgress the functional B genome *Ha* into CS. CS is genetically soft spring wheat that contains the *Pina-D1a* and *Pinb-D1a* alleles (Giroux and Morris 1997). We made a cross between CS#12 and the elite soft white spring wheat variety ‘Vanna’ (1995 release from WestBred LLC, Bozeman, MT). The F₂ plants were grown in a greenhouse at the Montana State University Plant Growth Center. Homozygous F₂ derived lines were identified as described below. The 72 F_{2:4} seed pools were planted in two 1.5 m rows in 2005 in both rain-fed and irrigated environments at the Arthur H. Post Agronomy Farm. Upon harvest, the individual rows were threshed, weighed, and then seed was combined across the two replications for each environment for grain quality testing.

Southern Blot

SKCS grain hardness testing was performed on the greenhouse produced F₂ derived F₃ seed from the cross between the substitution line CS#12 and the elite line ‘Vanna’. The five hardest and five softest F₂ derived F₃ lines were selected for Southern

blot hybridization. Genomic DNA was extracted from the selected lines and parents 'Vanna' as described in Riede and Anderson (1996). Genomic DNA was cut with *HindIII* and electrophoresed on a 6.0 x 9.25 cm 0.8% w/v 1.0X TBE agarose gel at 40 V for 4 hr. The remainder of the Southern blot hybridization was performed as described previously (Beecher et al. 2002).

Gsp and *Pinb* CAPS Markers

To develop a dominant *Pinb* Cleaved Amplified Polymorphic Sequence (CAPS) marker, *Pinb* sequence from the D genome of hexaploid wheat was compared with the *Pinb* sequence from the A genome diploid donor. *T. monococcum* *Pinb* sequence (AJ242716) and the *T. aestivum* D genome *Pinb* sequence (DQ363913) were aligned and polymorphic restriction sites were identified. The previously described *Pinb* primers PB5/PB3 (Gautier et al. 1994) were used to amplify the 447 bp *Pinb* coding sequence from each F₂ plant. The thermal cycling profile consisted of: 94°C for 3 min, then 40 cycles of 94°C 30 sec, 50°C 30 sec, 72°C 1 min, and a final extension at 72°C for 5 min. The *Pinb* PCR products were incubated with 2.5 units of *HphI* for 90 min at 37°C and then separated on a 2.5% Metaphor (Cambrex Bio Science, Rockland, ME, USA) agarose gel containing ethidium bromide and visualized with UV light.

To develop a codominant *Gsp* CAPS marker, primers were designed to amplify both the *T. monococcum* A genome *Gsp* sequence and the *T. aestivum* A genome *Gsp* sequence (AF177218) without amplifying the *T. aestivum* B or D genome *Gsp* sequences. Primers Gsp2F (5'GCCAAAGCTAGACTCTTGC3') and Gsp1R (5'ATCAATGTTGCACTTGGGA3') were used to amplify a 280 bp fragment from

each F_2 plant. The thermal cycling profile used consisted of: 94°C for 3 min, 40 cycles of 94°C 30 sec, 50°C 30 sec, 72°C 60 sec, and final extension at 72°C for 5 min. The *Gsp* PCR products were incubated with 2.5 units of *DdeI* for 90 min at 37°C and then separated as for the *Pinb* PCR products. All enzyme digests were performed on total PCR product and no purification steps were employed.

Grain Traits

Whole kernel protein and moisture content were measured using the near-infrared spectrophotometer Tecator Infratec 1225 (Foss North America Inc., Eden Prairie, MN) (Approved Method 39-25, AACC 2003). SKCS was performed on 50 seeds per sample using the Perten SKCS 4100 (Perten Instruments, Huddinge, Sweden) to determine kernel texture and seed size (Approved Method 55-31, AACC 2003).

Milling

The seed produced from the 2 *Ha* loci copy (2*Ha*) and 4 *Ha* loci copy (4*Ha*) $F_{2:4}$ homozygous individuals along with parents were milled on a Brabender Quadrumat Jr. flour mill (Brabender GmbH, Duisburg, Germany). Sample weights from both rain-fed and irrigated environments were between 70 g and 100 g prior to tempering and contained equal weights from each of the two replications. All samples were tempered to 14% moisture content and conditioned for 12-18 hr as per Approved Method 26-50 (AACC, 2003). Flour and bran yields were measured and total flour yield was calculated as (grams of flour)/ (total product: flour and bran). A homogenous 10.0 g weight of each sample was sifted on a RO-TAP RX-29 (Leval Lab Inc., Quebec, Canada) rotating sifter

through a series of four Seedburo (Seedburo Equipment Co., Chicago, IL) sieves of mesh sizes 0.053, 0.074, 0.149, and 0.30 mm for 10 min. Flour ash for each entry was measured as the average of duplicate determinations following Approved Method 08-01 (AACC 2003) where a 3.0 g sample of each flour is incinerated for 18 hr at 580 °C in porcelain crucibles. Flour ash is calculated as percentage ash = (weight of residue)/(weight of sample) x100. Starch damage for each entry was measured on white flour samples as the average of triplicate determinations using the Megazyme (Megazyme International, Bray, Co. Wicklow, Ireland) Damaged Starch kit as per AACC method 76-31 (2003).

Statistical Analysis

The *2Ha* versus *4Ha* group means were compared using a t-statistic. Correlations among traits were computed using entry means. All statistical analysis was performed using Microsoft Excel Windows XP Professional Software (Microsoft, Redmond, WA, U.S.A.).

Results

The ‘Chinese Spring’ chromosomal substitution line CS#12 created by See et al. 2004, contains functional copies of the *Pins* on both the A and B genome of hexaploid wheat. From the cross made between CS#12 and ‘Vanna’, 72 F₂ plants were produced displaying a range of grain hardness, ranging from 9.15 to 57.3, as measured by SKCS (Fig. 1).

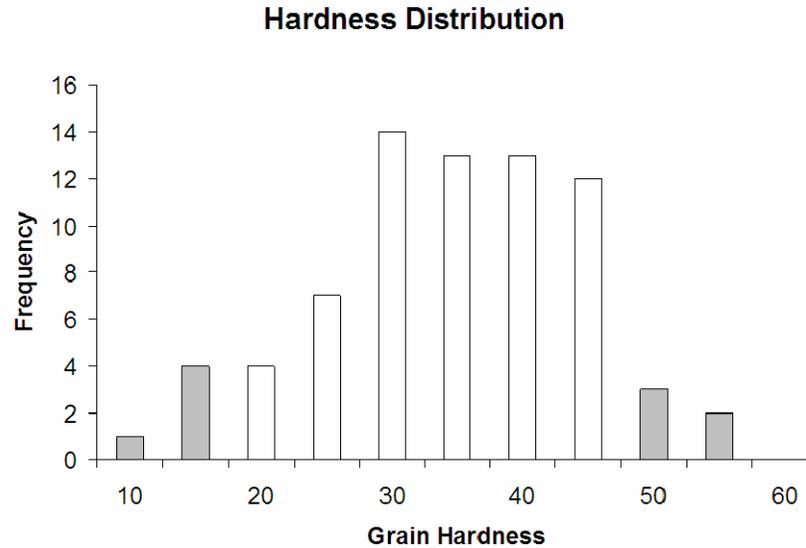


Figure 1. SKCS grain hardness distribution of the 72 F_2 individuals obtained from the cross of Vanna x CS#12. Five of the hardest and five of the softest individuals from the F_2 population were chosen for further investigation (shaded).

The *Ha* copy number in the five hardest and five softest F_2 individuals was elucidated via *Pinb* Southern blot hybridization. The Southern blots presented by See et al. (2004) indicated the relative positions of *Pinb* genome segments from largest to smallest; A, D, and B genome. The *Pinb* Southern blot performed indicates successful introgression of A genome *Pinb* in the five softest lines (Fig. 2) as denoted by the presence of the ~ 4.0 kbp band. The blot also demonstrates the presence of native D genome *Pinb* indicated by the band at ~ 2.5 kbp in all the lines indicating both successful inheritance of the A genome *Ha* locus as well as an apparent association with softer grain texture. The *Pinb* Southern blot indicates that there was no B genome *Ha* locus addition in any of the lines tested. Furthermore, there is no evidence of *Ha* locus addition to the A genome in the hardest lines.

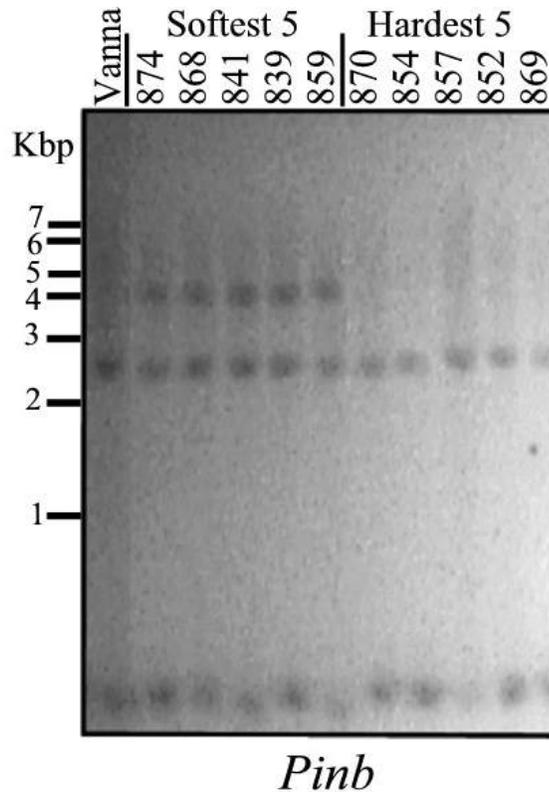


Figure 2. *Puroindoline b* Southern blot hybridization of leaf tissue extracted from F₂ plants with five of the softest and five of the hardest F₃ seed pools from the cross of Vanna x CS#12 and parents 'Vanna'. All lines contain a *Pinb* hybridizing band representing the native D genome *Pinb* at ~2.5Kb and the five softest F₂ exhibit the added A genome *Pinb* at ~4Kb.

The successful introgression of the A genome *Ha* segment catalyzed the development of a molecular marker based on difference between the native and alien *Pinb* sequences. Using Biology Workbench (<http://seqtool.sdsc.edu/CGI/BW>) a dominant marker was developed based on coding sequence comparison of *T. monococcum* A genome *Pinb* (A) (AJ242716) and *T. aestivum* D genome *Pinb* (D) (DQ363913) (Fig. 3). Sequence alignment identified a sequence difference that could be exploited by digesting the PCR product with *Hph*I.

<i>T. monococcum Pinb</i>	<u>ATGAAGGNCCTTATTCCCTCCTAGC</u> C CTCCTTGCTCTTGTAGCGAGCACAACTTCGCGCA
<i>T. aestivum Pinb</i>	<u>ATGAAG-ACCTTATTCCCTCCTAGC</u> T CTCCTTGCTCTTGTAGCGAGCACAACTTCGCGCA
<i>T. monococcum Pinb</i>	ATACTCAGAAGTTGGCGGCTGGTACAATGAAGTTGG T G CAGGAGGTGGTTCTCAACAATG
<i>T. aestivum Pinb</i>	ATACTCAGAAGTTGGCGGCTGGTACAATGAAGTTGG C G CAGGAGGTGGTTCTCAACAATG
<i>T. monococcum Pinb</i>	C CCGCT T GGAGCGGCCAAAGCTAAGCTCTTGCAAGGATTACGTGATGGAGCG G TGTTTCAC
<i>T. aestivum Pinb</i>	TCCGC A GGAGCGGCCGAAGCTAAGCTCTTGCAAGGATTACGTGATGGAGCG A TGTTTCAC
<i>T. monococcum Pinb</i>	AATGAAGGA-TTTTCCAGTCAC T TGGCCAC G AAATGGTGAAGGGCGG T TGTGAGCA C GA
<i>T. aestivum Pinb</i>	AATGAAGGA TTTTCCAG TCAC C TGGCCACA A AAATGGTGAAGGGCGG C TGTGAGCA T GA
<i>T. monococcum Pinb</i>	GGT C CGGGAGAAGTGCTG C CAGCAGCTGAGCCAGATAGCACCACAG T TGTCGCTG C AATTC
<i>T. aestivum Pinb</i>	GGT T CGGGAGAAGTGCTG A AAGCAGCTGAGCCAGATAGCACCACA A TGTCGCTG T GATTC
<i>T. monococcum Pinb</i>	TATCCG A G G A A TGATCCAAGGCA A GCTCGGTGGCTTCTT C G G AATTTGAAAGN GGT GAT G
<i>T. aestivum Pinb</i>	TATCCG G C G A G TGATCCAAGGCA G GCTCGGTGGCTTCTT G G C AATTTGGC- GAG GTGAG G
<i>T. monococcum Pinb</i>	CATTC AAACA A ATTCAGAGGGCCCAGAGCCTCCCCTCAAAGTGCAAACATGGG A GCCGAC
<i>T. aestivum Pinb</i>	TATTC AAACA C ATTCAGAGGGCCCAGAGCCTCCCCTCAAAGTGCAAACATGGG C GCCGAC
<i>T. monococcum Pinb</i>	TGCAA A TTCCTTAGTGGCTATTACTGGTGA
<i>T. aestivum Pinb</i>	TGCA G TTCCTTAGTGGCTATTACTGGTGA

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Figure 3. Comparison of *Pinb* sequences for *Triticum monococcum* (AJ242716) and *T. aestivum* (DQ363913). Primer sequences are underlined and sequence differences are boxed. *Hph*I recognition sites are in shaded boxes and cut sites are denoted with a break in the sequence with hyphens used as placeholders where the sequence remains uncut. The uncut fragment amplified with these primers is 447 bp. The *T. monococcum* sequence has 1 *Hph*I restriction site and is digested by the enzyme to yield fragments of sizes 84 and 363 bp. The *T. aestivum* sequence has 2 *Hph*I restriction sites; incubation with the enzyme yields 3 fragments of sizes 84, 175 and 188 bp.

<i>T. monococcum Gsp</i>	<u>GCCAAAGCTAGACTCTTGCAGCGATTATGTTATGGATCGGTGTGTGACGAAGGATATGCC</u>
<i>T. aestivum Gsp</i>	<u>GCCAAAGGTAGACTCTTGCAGCGATTATGTTATGGATCGGTGTGTGATGAAGGATATGCC</u>
<i>T. monococcum Gsp</i>	GCTCTCTTGGGTCTTTCC-TCGGACTTGGGGGAAGAGAAGTTGTGAGGAGGTCCAAAACCA
<i>T. aestivum Gsp</i>	GCTCTCTTGGTTCTTTCC TCAGACTTGGGGGAAGAGAAGTTGTGAGGAGGTCCAAAACCA
<i>T. monococcum Gsp</i>	GTGTTGTCAGCAATTGAGGCAAACAACGCCGCGTTGCCGCTGCAAGGCTATATGGACATC
<i>T. aestivum Gsp</i>	GTGTTGTAAGCAATTGAGGCAAACGACGCCGCGTTGCCGTTGCAAGGCTATATGGACATC
<i>T. monococcum Gsp</i>	AATCCAAGGCGAT CTAAGTGGCTTCAAGGGCCTTCAACAAGGTCTGAAGCCAAAACGGT
<i>T. aestivum Gsp</i>	AATCCAAGGCGAT CTAAGTGGCTTCAAGGGCCTTCAACAAGGTCTGAAGCCAAAATGGT
<i>T. monococcum Gsp</i>	GCAGACGGCCAAGAGCCTTCCCTCCAAGTGCAACATTGAT
<i>T. aestivum Gsp</i>	GCAGACGGCCAAGAGCCTTCCCTCCCAAGTGCAACATTGAT

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Figure 4. Comparison of *Gsp* sequences for *Triticum monococcum* A genome donor of CS#12 and *T. aestivum* A genome *Gsp* (AF177218). Primer sequences are underlined and sequence differences are boxed. *DdeI* recognition sites are in shaded boxes with cut sites denoted with a break in the sequence and hyphens used as placeholders. The uncut fragment amplified with these primers is 280 bp. The *T. monococcum* sequence has 1 *DdeI* restriction site and is digested by the enzyme to yield 2 fragments of sizes 87 and 193 bp. The *T. aestivum* sequence has 2 *DdeI* restriction sites; incubation with the enzyme yields 3 fragments of sizes 78, 87 and 115 bp.

Because there is not a *Pinb* sequence on the A and B genomes of common hexaploid wheat, we developed a codominant marker using the *Ha* locus component *Gsp* since *Gsp* is also present on the A, B and D genome of hexaploid wheat. The *Gsp* marker was developed by choosing *Gsp* primers specific to the A genome. *T. monococcum* A genome *Gsp* sequence and *T. aestivum* A genome *Gsp* sequence (AF177218) (Fig. 4) were aligned with the B and D genome *Gsp* sequences and the primers Gsp2F and Gsp1R were chosen to specifically amplify only A genome *Gsp* sequences. Further, restriction site analysis of diploid and hexaploid A genome *Gsp* sequences identified the enzyme *DdeI* as being able to distinguish between the two sequences. PCR was performed on parent and F₂ genomic DNA using primer pair PB5/PB3, and then the amplified products were digested with *HphI*. The presence of the introgressed diploid A genome *Pinb* in the population is indicated by two bands of lengths 84 and 363 bp (Fig. 5). The presence of the native hexaploid D genome *Pinb* is indicated by the presence of three bands of lengths 84, 175 and 188 bp. Sixty-one F₂ individuals were screened with the dominant *Pinb* marker and demonstrated a 21D:40A/D *Pinb* segregation ratio. For screening the genotypes, PCR was performed on genomic DNA using primer pair Gsp2F/Gsp1R, and the amplified 280 bp PCR products were digested with *DdeI*. The presence of the diploid A genome *Gsp* (A^m) was indicated by the presence of an 87 and a 193 bp band. The native hexaploid A genome *Gsp* (A) was indicated by the presence of bands of lengths 78, 87, and 115 bp. The heterozygous state was indicated by the presence of four bands of lengths 78, 87, 115, and 193 bp. The genome and copy number of the *Ha* locus will be described as first denoted by See et al. (2004); the native hexaploid A genome *Ha*

segment designated as “A” and the introgressed diploid A genome designated as “A^m”, with apostrophes indicating copy number. Sixty-four F₂ individuals were screened with the codominant *Gsp* marker and they segregated as 21A'' : 22 A'/A^m : 21A^m'. Seed collected from plants found to be homozygous for the presence (A^m' or 4*Ha*) or absence (A'' or 2*Ha*) of the added A genome *Ha* locus were advanced for grain quality testing.

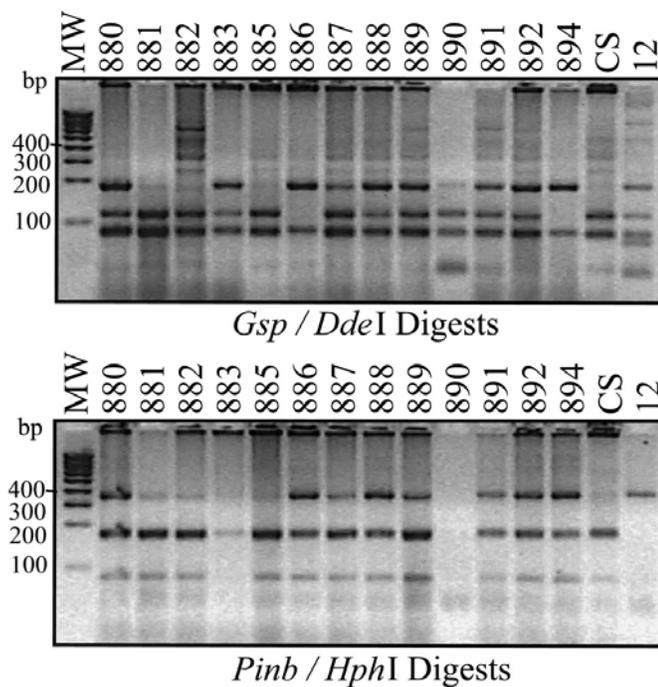


Figure 5. Genotyping of F₂ population (881-894) and parents ‘Chinese Spring’ (CS) and CS #12 (12). The 280 bp *Gsp* PCR products were incubated with restriction endonuclease *DdeI*. CS represents the homozygous negative class with 2 *DdeI* cut sites yielding 3 bands of sizes 78, 87, and 115 bp; 12 represents the homozygous positive class with 1 *DdeI* cut site yielding 2 bands of sizes 87 and 193 bp. Lane 880 is heterozygous for the additional segment with 4 bands of sizes 78, 87, 115 and 193 bp. The *Pinb* 447 bp PCR products incubated restriction endonuclease *HphI*. CS represents homozygous negative class with 2 cut sites yielding 3 bands of sizes 84, 175, and 188 bp; 12 represents the homozygous positive class with 1 *HphI* cut site yielding bands of sizes 84 and 363 bp. Lane 880 represents the segregating homozygous positive and the heterozygous class because the *Pinb HphI* marker is dominant and therefore contains bands representing both the D genome *Pinb* as well as the A genome *Pinb* with 3 *HphI* cut site yielding 4 bands of sizes 84, 175, 188 and 363 bp.

Data collected on seed grown in the greenhouse in 2004 and the field in 2005 indicated consistent change in kernel characteristics of the lines with 4 copies of the *Ha* locus (*4Ha*) compared to lines with only the two native copies of the *Ha* locus (*2Ha*) (Table 1). SKCS grain hardness was performed on the seed grown in the greenhouse in 2004 using the F₂:F₃ seeds. The *4Ha* group was 8.76 hardness units softer than the *2Ha* group. Similar results were obtained using the F_{2,4} seed from plants grown in the field in 2005 where the *4Ha* group was 7.4 units softer than the *2Ha* lines. The SKCS grain hardness values for the three parents CS, ‘Vanna’, and disomic substitution line parent (+dsA^m) were measured at 42.9, 11.3 and 16.7, respectively. There was a decrease in grain yield in the *4Ha* lines with a 3.81% reduction in mean yield weight compared to the *2Ha* lines. Small-scale milling, between 70 g and 100 g starting seed weight, was performed with a Quadrumat Brabender Jr. mill. Milling and flour traits showed significant differences between lines varying in *Ha* copy number (Table 2). In particular, flour yield and ash content, as well as particle size distribution of flour were affected. Overall flour yield decreased by 3.4% in the *4Ha* lines compared to the *2Ha* lines. Percentage flour ash decreased as well in the *4Ha* lines by 0.02% compared to the *2Ha* lines. One of the most significant changes in flour quality associated with the increase in *Ha* dosage was flour particle size distribution. The flour from the lines containing the additional copies of *Ha* had particles of smaller average size. In the *4Ha* lines the percentage of medium sized particles (0.074 to 0.149 mm) decreased by 5.92% relative to the *2Ha* lines and the percentage in the smallest size range, <0.053 mm, increased by

4.46% compared to the *2Ha* lines. Damaged starch content proved to be highly associated with hardness as the percentage of damaged starch in the *4Ha* lines was reduced by 0.35% as compared to the *2Ha* lines. The grain hardness reduction inherent in the *4Ha* lines was correlated with several flour traits (Table 3).

Damaged starch was positively correlated with grain hardness. The percentage of particles in the 0.074 to 0.149 mm range correlated positively with increasing hardness and the percentage of particles <0.053 mm decreased with increasing hardness. To best illustrate the correlation between damaged starch and SKCS grain hardness the results were graphed (Fig. 6A). As damaged starch increased so did grain hardness in comparisons of lines with two versus four *Ha* loci. Also visually apparent is the relationship between grain hardness and flour composition as mean increase in percentage of flour particles in the 0.074 to 0.149 mm range was correlated with increasing grain hardness (Fig. 6B)

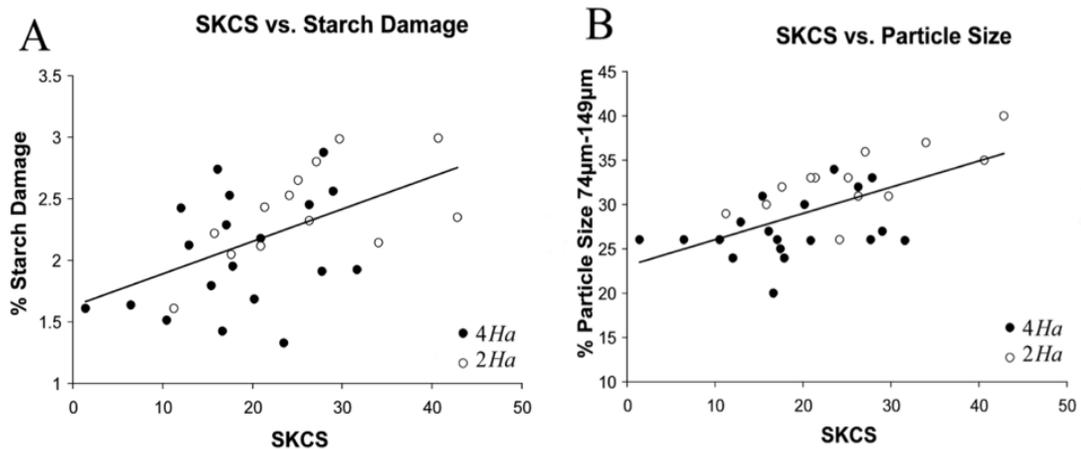


Figure 6. Relationship between grain hardness and starch damage and flour particle size. A. Correlation between the presence of added A genome *Ha* locus and the percentage of starch damage. B. Correlation between presence of added A genome *Ha* locus and percentage of flour particles 0.149-0.074 mm. *4Ha* represents lines homozygous for the added A genome *Ha* locus. *2Ha* represents lines with only native copies of *Ha* locus.

Population ^a	<i>n</i> ^b	2004		2005			
		Grain Hardness ^c	Kernel Weight (mg) ^d	Grain Hardness ^c	Kernel Weight (mg) ^f	Protein Content (g/kg) ^g	Agronomic Yield (g) ^h
4 <i>Ha</i>	21	28.0 (2.4)	39.2 (1.0)	18.5 (1.8)	31.0 (0.51)	158.0 (2.9)	164.1 (9.7)
2 <i>Ha</i>	22	36.8 (2.7)	39.3 (1.3)	25.9 (2.6)	30.8 (0.36)	152.0 (3.0)	170.6 (10.4)
<i>P</i>		0.0087	0.4	0.01	0.39	0.15	0.03
CS	1			42.9 (2.5)	28.9 (0.38)	164	43.0 (4.1)
Vanna	1			11.3 (3.1)	30.5 (0.38)	139	174.4 (6.6)
+dsAm	1			16.7 (0.39)	25.9 (0.12)	185	90.4 (24.3)

Table 1. Effects of Added *Ha* Locus Upon Kernel Traits. Comparative statistics of F_{2:4} population including parents.

^a Population 4*Ha* represent recombinant F_{2:4} progeny that carry the added A genome *Ha* locus in addition to the native D genome *Ha* locus.

2*Ha* represent recombinant F_{2:4} progeny that carry only native D genome *Ha* locus. Mean values for lines (standard error). Parents ‘Chinese Spring’ (CS), ‘Vanna’, and ‘Chinese Spring’ #12 disomic substitution line (+dsAm).

^b Sample group size.

^c Greenhouse population data for 2004 F₃ seed from single F₂ plants. Grain hardness (standard error) for each homozygous class.

^d Kernel weight means (standard error) for each homozygous class grown in 2004.

^e Field population data for 2005 F_{2:4} seed. Grain hardness means (standard error) for each homozygous class and parents.

^f Kernel weight means (standard error) for each homozygous class and parents grown in 2005.

^g Protein content means (standard error) for each homozygous class and parents, as measured by NIR (g/kg).

^h Total agronomic yield means (standard error) for each homozygous class and parents, measured as total grain weight (g) obtained from each field row in 2005.

Population ^a	Flour Yield (%) ^b	Starch Damage (%) ^c	Flour Ash (%) ^d	% Total Recovery			
				Size of Flour Particles (mm) ^e			
				>0.149	0.149-0.074	0.073-0.053	<0.053
<i>4Ha</i>	69.64 (0.65)	2.05 (0.10)	0.53 (0.011)	23.96 (0.65)	27.65 (0.79)	11.39 (1.10)	37.00 (1.48)
<i>2Ha</i>	73.04 (0.61)	2.40 (0.010)	0.55 (0.018)	23.40 (0.74)	33.57 (1.01)	10.49 (0.95)	32.54 (1.90)
<i>P</i>	<0.001	0.02	0.05	0.23	<0.001	0.27	0.03

Table 2. Milling Traits and Flour Particle Size Distribution. Comparative statistics associated with flour traits measured from F_{2:4} population.

^a Population *4Ha* represent recombinant F_{2:4} progeny that carry the added A genome *Ha* locus in addition to the native D genome *Ha* locus. *2Ha* represent recombinant F_{2:4} progeny that carry only native D genome *Ha* locus.

^b Total flour yield as a percentage of total milling product recovered (standard error) for each homozygous class.

^c Percentage starch damage (standard error) for each homozygous class.

^d Flour ash for each homozygous class including (standard error).

^e Particle size given as a percentage of 10 g sample sifted with a minimum of 95% return (standard error) for each homozygous class. Size ranges given in (mm).

Traits	Grain Hardness ^b	Kernel Protein ^c	Bran Yield	Flour Yield	Particle Size (mm) ^d				Starch Damage ^e
					>0.149	0.149-0.074	0.073-0.053	<0.053	
Kernel Protein	0.08								
Bran Yield	-0.38*	0.39*							
Flour Yield	0.35*	-0.40*	-0.94***						
>0.149	0.33*	0.41**	0.22	-0.08*					
0.149-0.074	0.61**	-0.15	-0.80***	0.75**	-0.01				
0.073-0.053	-0.16	0.40*	0.51***	-0.43*	0.45***	-0.33*			
<0.053	-0.41**	-0.35*	0.13	-0.19*	-0.65***	-0.41**	-0.65***		
Starch Damage	0.52***	-0.25	-0.25	0.18*	-0.15	0.34*	-0.21	-0.01	
Flour Ash	0.71***	0.32*	-0.33*	0.23*	0.17	0.41**	-0.23	-0.22	0.17

Table 3. Relationship Between SKCS Grain Hardness and Associated Kernel and Flour Traits.

^a *, **, *** Statistical significance at 5, 1, and 0.5 %.

^b SKCS grain hardness as measured by Perten SKCS 4100.

^c Whole kernel protein as measured by NIR (g/kg).

^d Flour particle size ranges of fractioned flour obtained from the Quadrumat Jr. Mill (mm).

^e Percentage damaged starch as measured using the Megazyme Damaged Starch kit.

Discussion

Grain texture is a determining factor in the end product use of commercial wheats, as hard wheats are used for baking breads and soft wheats are used for baking pastries (reviewed by Morris and Rose 1996). Associated with the variation in hardness are important kernel and flour qualities such as flour yield, flour particle size, starch damage and water absorption (Symes 1969; Moss et al. 1973; Rogers et al. 1993). Soft wheats have reduced flour yield on small-scale mills, and produce flours with a smaller mean particle size, reduced starch damage and lower water retention capacity when compared to hard wheats (Symes 1969; Rogers et al. 1993). The *Ha* locus, which contains *Pina*, *Pinb* and *Gsp*, is the primary locus that controls wheat grain texture (Sourdille et al. 1996; Giroux and Morris 1998; Jolly et al. 1993; Symes 1964). Active *Pins* reside on the D genome of commercial hexaploid wheat and in the diploid A and B genome progenitors (Gautier et al. 2000; Chantret et al. 2005). Of the three genes present at the *Ha* locus, only *Pina* and *Pinb* have been functionally linked to grain hardness variation. Any mutation in the coding sequence of either of the *Pin* genes results in a hard phenotype (Giroux and Morris 1997; Giroux and Morris 1998).

It was demonstrated that *Pin* mutations are causative to hard wheat phenotypes when soft texture was achieved as a result of complementation of hard phenotypes with wild-type *Pin* alleles (Beecher et al. 2002; Martin et al. 2006). Together, PINA and PINB constitute the starch surface protein complex friabilin, which is present at high levels in soft wheats and low levels in hard wheats (Greenwell and Schofield 1986). The variation

between soft and hard textured wheats can be attributed mostly to sequence divergence in the *Pin* genes (Giroux and Morris 1997; Giroux and Morris 1998). The variation among soft wheat varieties cannot be attributed to *Pin* sequence variation as all soft wheats have the same *Pin* alleles (Giroux and Morris 1998; Morris and Lillemo 2000). The introduction of additional copies of functional *Puroindolines* on the A and B genomes has been observed to reduce mean hardness by 10 SKCS units per additional *Ha* locus in a relatively hard textured soft wheat background by See et al. (2004). Here we demonstrate a similar mean reduction, 7.4 SKCS units, in hardness with *Ha* overexpression in an elite soft wheat. The data presented here demonstrates that increased *Ha* copy number from substitution-line introgression has an observable effect on kernel and flour end product qualities important for soft wheat use. The flour qualities associated with the observed reduction in hardness includes reduced flour yield, reduced starch damage and reduced flour ash content. Reduced starch damage is associated with the desirable increase in sugar-snap cookie diameter (Rogers et al. 1993). The flour composition was affected as the increased dosage of *Pins* led to a shift in particle size towards smaller particles. The overexpression of *Ha* in the soft background resulted in a reduction in kernel weight and a reduction in overall agronomic yield. The reduction in total agronomic yield is most likely a genetic background artifact as the lines reported here are only 50% elite background with 50% of the background comprised of lesser quality genes from CS which is not well adapted to Montana growing conditions. This successful, stable introgression of the functional A genome *Ha* into ‘Vanna’ with

significant changes in flour quality indicates that a very soft wheat line is possible in commercial production and may yield flour with added value.

Conclusion

This study demonstrates that alien segments from the diploid A genome donor, *T. monococcum*, of hexaploid wheat can be non-transgenically and stably introgressed into hexaploid genome via chromosomal substitution lines. This introgression leads to positive significant changes in the end product quality traits desired in soft wheat flours. The overexpression of functional *Pins* leads to an increase in fine particle size, reduced starch damage, and reduced flour ash content which was associated with reduced flour yield on the small scale mill used in this study.

CHAPTER 2

END PRODUCT QUALITY EFFECTS OF NOVEL *Ha* LOCI FROM *TRITICUM*
TAUSCHII L. ON SOFT WHEAT (*TRITICUM AESTIVUM* L.)Introduction

Grain texture is one of the most important characteristics that determines milling and end-product qualities of wheat (*Triticum aestivum* L.). All commercial hexaploid wheats are categorized as either “hard” or “soft” based on kernel texture. The properties associated with hard kernel texture render hard wheats better suited for making bread and soft wheats better suited for making pastries (Reviewed in Morris and Rose 1996). Some of the important flour properties affected by grain texture include flour yield, water absorption, particle size, and percent starch damage (Symes 1964; Rogers et al. 1993). Soft wheats have lower flour yield with lower water retention capacities, smaller mean particle size, and lower levels of damaged starch when compared to hard wheats (Symes 1964; Rogers et al. 1993). Among the distinctive properties associated with soft wheat quality, damaged starch particles are of particular interest in soft wheats as damaged starch affects sugar-snap cookie spread with lower damaged starch resulting in greater cookie diameter (Rodgers et al. 1993).

Wheat grain texture is simply inherited and controlled by the *Hardness* (*Ha*) locus (Symes 1964). The *Ha* locus consists of the *Puroindoline a* (*Pina*) and *Puroindoline b* (*Pinb*) genes (Sourdille et al. 1996; Giroux and Morris 1998) with soft texture (*Ha*)

dominant to hard (*ha*) (Baker 1977; Law et al. 1978). *Ha* has been localized to the distal end of chromosome 5D (5DS) in hexaploid wheat (Mattern et al. 1973). Though the *Gsp* gene is physically linked to the *Pin* genes; variation in *Gsp* copy number does not affect grain texture (Tranquilli et al. 1999). In addition, there have been no studies that link *Gsp* allelic differences to grain texture differences.

The causative relationship between *Pins* and grain texture was confirmed with studies that demonstrated functional complementation of the most common *Pina* and *Pinb* mutations with wild-type *Pin* alleles (Beecher et al. 2002, Martin et al. 2006). Both PINs have been shown to affect grain texture in soft wheats, though PINB has been demonstrated to limit grain softness more than PINA (Swan et al. 2006). Transgenic overexpression of wild-type *Pins* in a hard background demonstrated significant end product and milling effects (Hogg et al. 2005). Non-transgenic studies have demonstrated that additional *Ha* loci introduced through chromosomal substitution lines, decreased grain hardness in hexaploid wheat (See et al. 2004; Campbell et al. 2007). The end product quality traits shown to be affected by transgenic and non-transgenic PIN overexpression include flour yield, break flour yield, flour ash, starch damage, and bread loaf volume (Hogg et al. 2005; Campbell et al. 2007). The presence of wild-type *Puroindolines*, *Pina-D1a* (PINAa) and *Pinb-D1a* (PINBa), is associated with a soft kernel phenotype, and a mutation in either *Pina* or *Pinb* produces a harder phenotype (Giroux and Morris 1997).

PINA and PINB associated with starch granules are referred to collectively as friabilin. This complex was described by Greenwell and Schofield (1986) as a 15kDa

protein complex on the surface of water-washed starch granules. Specifically, friabilin is found at higher levels on soft wheat starch compared to hard wheat starch and is absent from durum wheats (Greenwell and Schofield 1986). A more precise molecular mass measurement of the PINAa protein was obtained through electro-spray mass spectrometry where it was measured at 12.8 kDa (Blochet et al. 1993). Based on amino acid composition the mass of PINBa is estimated to be 13.1 kDa.

The *Ha* locus is exceptional in that it is a single locus considering that bread wheat is an allohexaploid ($2n = 6x = 42$). The evolutionary path of modern hexaploid wheat involved primary speciation and secondary hybridization of the diploid progenitor species *Triticum urartu* ($2n = 2x = 14$ AA) and *Aegilops searsii* ($2n = 2x = 14$ BB) (McFadden and Sears 1946). This hybridization event resulted in the tetraploid durum wheat, *T. turgidum* ($2n = 4x = 28$ AABB). A subsequent hybridization event combined *T. turgidum* (AABB) with *Aegilops tauschii* ($2n = 2x = 14$ DD) producing modern hexaploid wheat *T. aestivum* (AABBDD) (McFadden and Sears 1946). A *Ha* locus is found in progenitor diploid species and hexaploid bread wheat (Gautier et al. 2000; Chantret et al. 2005). However, functional *Puroindolines* are found only on the D genome of hexaploid wheat and are entirely absent from the tetraploid A and B genomes (Gautier et al. 2000; Chantret et al. 2005).

The rare and relatively recent hybridization event that resulted in *T. aestivum* has led to intense genetic constraints on the level of genetic diversity among *T. aestivum* wheat varieties. *Pin* sequence surveys conducted using diploid wheats indicate there is substantial D genome *Pin* allelic diversity that could be utilized to introduce *Pin* variation

into hexaploid wheat via crossing locally adapted wheats with synthetic hexaploid lines (Massa et al. 2004; Gedye et al. 2004; Chen et al. 2005). A *Ha* locus survey by Massa et al. (2004) identified the *Pina* and *Pinb* alleles present in fifty *Ae. tauschii* subsp. *tauschii* and *Ae. tauschii* subsp. *strangulata* accessions. Among the fifty *Ae. tauschii* accessions, four novel *Pina* alleles which translated into two unique peptides, and four novel *Pinb* alleles which translated into three unique peptides (Massa et al. 2004) were identified. Another survey of fifty *Ae. tauschii* species by Chen et al. (2005) uncovered three more novel *Pinb* alleles and six novel *Pina* alleles. Though Chen et al. 2005 inferred *Pin* allelic variation was associated with grain texture differences through freeze fracture Scanning Electro Microscopy (SEM), there is admitted difficulty in demonstrating the effects of the *Pins* within the *Aegilops* genetic background.

Expanding upon the investigation of novel synthetic alleles present among *Ae. tauschii* accessions; Gedye et al. (2004) looked at *Pina* and *Pinb* sequences present among seventy-five synthetic hexaploid wheat accessions. The seventy-five synthetic accessions evaluated revealed eight *Pina* alleles which translated to four unique peptides and seven *Pinb* alleles which translated to two unique peptides (Gedye et al. 2004). In these synthetic lines the impact of the allelic state of the *Puroindolines* upon grain hardness was evaluated. A mean grain hardness reduction of 9.3 units was observed in synthetic lines carrying PINAc compared to synthetic lines carrying PINAa, and synthetic lines carrying PINBh were 4.6 units softer than those carrying PINBa. This textural variation, however, could not be separated from the effects of the synthetic background in

the lines evaluated as there are many minor genes that contribute to kernel texture (Anjum and Walker 1991) in these backgrounds. Therefore, a linkage between soft wheat kernel texture and the novel *Pin* alleles has not been proven.

Here we evaluate the end product quality effects of select novel *Pin* alleles in a hexaploid background via analysis of progeny isolated from crosses between synthetic wheats and the Montana adapted soft white spring wheat ‘Vanna’ (Westbred Co., Bozeman, MT). To determine the best haplotype candidates for evaluation a total PIN content survey was conducted using eighteen select elite synthetic lines. Two *Ha* locus haplotypes were selected for investigation based on preliminary data indicating that the genotypes may have significant phenotypic effects (Gedye et al. 2004) and PIN levels identified in initial germplasm screening. The *Ha* loci selected are *Pina-D1e* / *Pinb-D1i* expressing the proteins PINAc / PINBh and *Pina-D1a* / *Pinb-D1j* expressing the proteins PINAa / PINBj. The translated protein PINAc has one amino acid substitution compared to PINAa whereas PINBh and PINBj have many amino acid substitutions compared to PINBa. The amino acid substitution that was speculated by Gedye et al. (2004) to impart a softer phenotype is the change of position 28 from an arginine (R) to a tryptophan (W), and is shared between PINBh and PINBj.

Materials and Methods

Genetic Material

The synthetic elite hexaploid wheat lines were created at CIMMYT and obtained from the Wheat Genetics Resource Center at Kansas State University. The *Ha* locus

Pina-D1e / *Pinb-D1i* is represented by elite synthetic line 14 (WGRC Elite14) with the *Ae. tauschii* parentage YUK donor TA2462 and identified as WGRC cross CIGM90-561. The *Ha* locus *Pina-D1a* / *Pinb-D1j* is represented by elite synthetic line 22 (WGRC Elite22) with the *Ae. tauschii* parentage D67.2 / P66.270 donor accession TA1599 and identified as WGRC cross CIGM88.1216-0B (<http://www.kstate.edu/wgrc/Germplasm/ELITE97>). The synthetic lines were crossed with the elite soft white spring wheat ‘Vanna’, a 1995 release from Westbred (Westbred Co., Bozeman, MT). Two independent crosses were made between ‘Vanna’ and synthetic line 14 which produced eight F₁ seeds. Four independent crosses were made between ‘Vanna’ and synthetic line 22 which produced eighteen F₁ seeds. All F₁ seed was grown in the Plant Growth Center at Montana State University, Bozeman and the F₂ seed from each F₁ plant was harvested in the spring of 2005. The F₂ seed was grown at the Arthur H. Post Agronomy Farm planted at a rate of twenty seeds per row with each row being the product of a single F₁ plant. Eight rows per cross along with two rows of each parent were grown in the spring of 2005. The F₃ seed from each F₂ plant was harvested in the summer of 2005.

Subsamples of twenty seeds from each of the F₃ seed pools were genotyped to identify homozygous lines, by the USDA-ARS genotyping lab in Pullman, WA using the Codominant Cleaved Amplified Polymorphic Sequence (CAPS) markers as described below. The F₂ segregation ratios based on the F₂ derived F₃ seed pools were 33 *Pinb-D1i* / *Pinb-D1i* : 38 *Pinb-D1i* / *Pinb-D1a* : 27 *Pinb-D1a* / *Pinb-D1a* ($\chi^2 = 5.67$ P = 0.001) for the Vanna x Elite14 population and 26 *Pinb-D1j* / *Pinb-D1j* : 47 *Pinb-D1j* / *Pinb-D1a* :

29 *Pinb-D1a* / *Pinb-D1a* ($\chi^2 = 0.8$ P = 0.37) for the Vanna x Elite22 population. F_{2.4} seed homozygous for the *Ha* locus from each population plus parents and ‘Chinese Spring’ were planted in two replications of a randomized block design in 1.5 m rows spaced 30 cm apart. Each population was planted as separate adjacent experiments in both rain-fed and irrigated environments at the Arthur H. Post Agronomy Farm in the spring of 2006. Upon harvest, the individual rows were threshed, weighed, measured for grain hardness and then grain was combined across the two replications for each environment separately for grain quality testing.

Pinb CAPS Markers

Codominant Cleaved Amplified Polymorphic Sequence (CAPS) markers were developed for both populations based on sequence comparisons between *Pinb-D1a* (DQ363913), *Pinb-D1i* (AY251962), and *Pinb-D1j* (AY251972). The *Pinb* sequences were aligned and polymorphic restriction enzyme sites were identified. The previously described *Puroindoline b* coding region primers PB5/PB3 (Gautier et al. 1994) were used to amplify a 447 bp fragment. The thermal cycling profile used consisted of one initial extension cycle of 94 °C for 3 min, 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min, and one final extension cycle of 72 °C for 5 min. The *Pinb* coding sequence PCR products were incubated for 90 min with 2.5 units of *Bst*NI at 60 °C for Vanna x Elite14 lines or with 2.5 units of *Hpa*II at 37 °C for Vanna x Elite22 lines. The digested fragments were separated on a 1.5 % agarose gel containing ethidium bromide and visualized with UV light.

Grain Trait Analysis

Whole kernel protein and moisture contents were measured using a Tecator Infratec 1225 near-infrared spectrophotometer (Foss North America Inc., Eden Prairie, MN) (Approved Method 39-45, AACC 2003). Grain hardness measurements were performed on pools of fifty seeds per sample using a Perten SKCS (Single Kernel Characterization System) 4100 (Perten Instruments, Springfield, IL) (Approved Method 55-31, AACC 2003).

Flour Quality Analysis

Each 50 g sample containing equal weights from the two replications from either rain-fed or irrigated environments was milled on a Brabender Quadrumat Jr. flour mill (Brabender GmbH, Duisburg, Germany). Samples were tempered at 14 % moisture content for 18-24 hrs prior to milling (Approved Method 26-50, AACC 2003). The recovered flour and bran fractions were weighed and total flour yield was calculated as $(\text{grams of flour} / \text{grams total product}) \times 100$. Flour samples recovered were mixed until homogenous, and a 10 g sample of each was sifted on a RO-TAP RX-29 rotating sifter (Leval Lab Inc., Quebec, Canada) through a series of four Seedburo sieves (Seedburo Equipment Co., Chicago, IL) of mesh sizes 0.053, 0.074, 0.149, and 0.30 mm for 10 min. Flour ash content was measured in duplicate and averaged for each replication of each line by incineration for 18 hr at 580 °C of a 3.0 g flour sample in porcelain crucibles (Approved Method 08-01, AACC 2003). Flour starch damage was measured on duplicate 100 mg samples and averaged for each replication of each line using a Megazyme

Damage Starch kit from (Megazyme International, Bray, Co. Wicklow, Ireland) (Approved Method 76-31, AACC 2003). Flour protein was measured on duplicate 2.0 g samples and averaged for each line using a Tecator Infratec 1225 near-infrared spectrophotometer (Foss North America Inc., Eden Prairie, MN) (Approved Method 39-11, AACC 2003).

Puroindoline Isolation, MALDI-TOF, and SDS-PAGE

Total PIN fractions were obtained through Triton X-114 phase partitioning as described by Giroux et al. (2003) with a starting weight of 100 mg of flour obtained from the small-scale milling defined above. The isolated fractions were suspended in 20 μ L volume of 50 % v / v acetonitrile / water containing 0.1 % formic acid. A 3.0 μ L volume of each protein sample was spotted on an aluminum plate and analyzed using a Bruker Biflex III Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (Bruker Daltonics, Billerica, MA). Four randomly chosen lines from each homozygous class and parents were analyzed. The mean value for each genotype was obtained from the average of the four lines for each homozygous class. A constant laser power of 62 % was maintained for all ionizations. A standard curve was created for Cytochrome *c* and wild-type soft *Puroindoline* (Heron) controls. Linear regressions were calculated for each standard curve and all data was fit to the regression equation obtained from Cytochrome *c* linear regression. The same lines analyzed above were fragmented on Cambrex 10-20 % SDS-PAGE mini gels (Cambrex, East Rutherford, NJ) at 125 V for 45 min and stained

with 0.1 % Coomassie stain for 3 hrs. After a 2 hr destain the gels were visualized on a florescent light box.

Statistical Analysis

All mean values were compared using a t-statistic in Microsoft Excel Windows XP Professional Software (Microsoft, Redmond, WA, U.S.A.). Allelic class mean values were compared with a two-tailed t-statistic where the entry within class variation was used as the error.

Results

The synthetic wheat collections contained at the International Maize and Wheat Improvement Center (CIMMYT) were screened for synthetic hexaploid wheat lines that had *Ha* loci differing from haplotypes represented in the adapted *T. aestivum* germplasm. Eighteen lines of varying *Pin* allelic state were chosen among the elite synthetic accessions available (Table 4). The selected eighteen elite synthetic lines with differing *Ha loci*, demonstrated significant variation in total PIN levels as evaluated via TX-114 extraction of whole seed meals (Fig. 7). Based on the previously published surveys and total PIN levels two haplotypes were chosen out of the eighteen represented for further investigation. The data from Gedye et al. (2004) suggested the *Pina-D1e / Pinb-D1i Ha* locus would have reduced grain hardness, and the total PIN preparations from synthetic elite line Elite 14 indicated increased levels of PIN compared to a soft wheat control. Gedye et al. (2004) also suggested that the *Pina-D1a / Pinb-D1j Ha* locus would impart no significant change in grain texture, and the synthetic total PIN preparation of the

synthetic line Elite 22 indicated a similar level of PIN as the *Pina-D1e / Pinb-D1i Ha* locus synthetic and would therefore serve as a control.

The PINAc mature protein amino acid sequence is compared to the PINAa in Figure 8 and the PINBh and PINBj mature protein amino acid sequences are compared to PINBa in figure 9. The translated protein PINAc has one amino acid substitution that distinguishes it from PINAa. The translated protein PINBh has a total of thirteen amino acid substitutions relative to protein PINBa and the translated PINBj has a total of nine amino acid substitutions versus PINBa.

Parents ^a	<i>Pina</i> Allele ^c	PINA Allele ^d	<i>Pinb</i> Allele ^c	PINB Allele ^f	<i>Ae. tauschii</i> Accession ^g
Vanna	<i>Pina-D1a</i>	PINAa	<i>Pinb-D1a</i>	PINBa	TA2460
McNeal	<i>Pina-D1b</i>	NULL	<i>Pinb-D1a</i>	PINBa	
Canadian Red	<i>Pina-D1a</i>	PINAi	<i>Pinb-D1e</i>	NULL	
Elite Accession ^b					
TA4152 L22 (elite#22)	<i>Pina-D1a</i>	PINAa	<i>Pinb-D1j</i>	PINBj	TA1599
TA4152 L14 (elite#14)	<i>Pina-D1e</i>	PINAc	<i>Pinb-D1i</i>	PINBh	TA2462
TA4152 L57 (elite#57)	<i>Pina-D1a</i>	PINAa	<i>Pinb-D1a</i>	PINBa	TA2460
TA4152 L65 (elite#65)	<i>Pina-D1d</i>	PINAc	<i>Pinb-D1i</i>	PINBh	TA2394
TA4152 L29 (elite#29)	<i>Pina-D1a</i>	PINAi	<i>Pinb-D1i</i>	PINBh	TA2475
TA4152 L47 (elite#47)	<i>Pina-D1d</i>	PINAc	<i>Pinb-D1i</i>	PINBh	TA2455
TA4152 L13 (elite#13)	<i>Pina-D1e</i>	PINAa	<i>Pinb-D1i</i>	PINBh	TA2462
TA4152 L26 (elite#26)	<i>Pina-D1c</i>	PINAc	<i>Pinb-D1h</i>	PINBh	TA2454
TA4152 L73 (elite#73)	<i>Pina-D1c</i>	PINAc	<i>Pinb-D1h</i>	PINBh	TA2470
TA4152 L74 (elite#74)	<i>Pina-D1c</i>	PINAc	<i>Pinb-D1h</i>	PINBh	TA2468
TA4152 L46 (elite#46)	<i>Pina-D1c</i>	PINAc	<i>Pinb-D1h</i>	PINBh	TA2452
TA4152 L72 (elite#72)	<i>Pina-D1c</i>	PINAc	<i>Pinb-D1h</i>	PINBh	TA2450
TA4152 L86 (elite#86)	<i>Pina-D1a</i>	PINAa	<i>Pinb-D1j</i>	PINBj	TA2531
TA4152 L39 (elite#39)	<i>Pina-D1d</i>	PINAc	<i>Pinb-D1o</i>	PINBh	TA1618
TA4152 L34 (elite#34)	<i>Pina-D1j</i>	PINAj	<i>Pinb-D1i</i>	PINBi	WX511
TA4152 L26 (elite#26)	<i>Pina-D1c</i>	PINAc	<i>Pinb-D1h</i>	PINBh	TA2454
TA4152 L17 (elite#17)	<i>Pina-D1a</i>	PINAa	<i>Pinb-D1h</i>	PINBh	TA2470
TA4152 L7 (elite#7)	<i>Pina-D1h</i>	PINAa	<i>Pinb-D1i</i>	PINBh	WX205

Table 4. List of Elite Synthetic Alleles, Proteins and Accession Numbers. Including wild-type and null controls.

^a Parent controls.

^b WGRC elite synthetic accession designation.

^c *Pina* gene designation for each entry.

^d PINA protein encoded for by specific allele.

^e *Pinb* gene designation for each entry.

^f PINB protein encoded for by specific allele.

^g *Ae. tauschii* parent donor for each synthetic line.

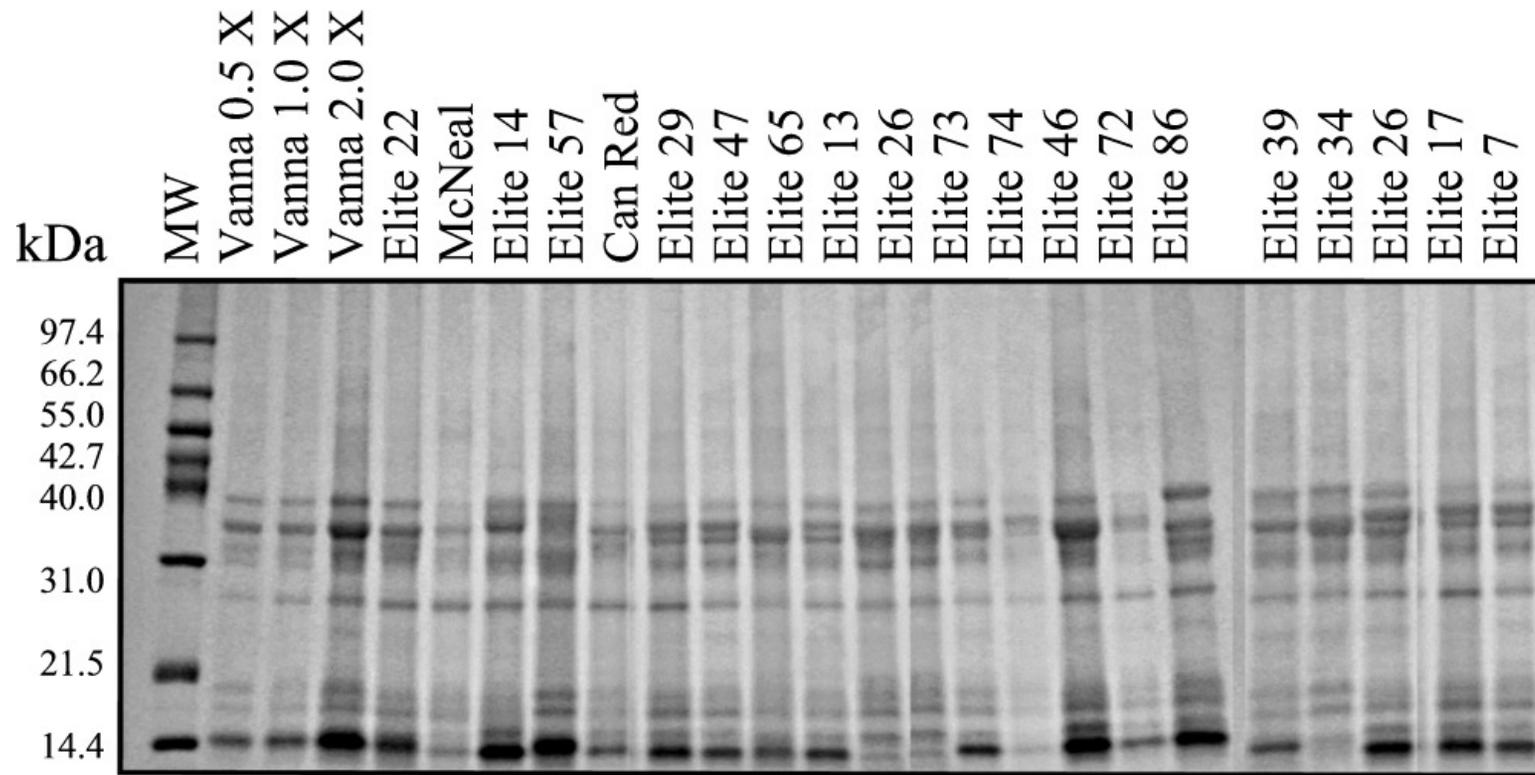


Figure 7. SDS-PAGE gel of TX-114 isolation of total PIN from each line selected for investigation in elite synthetic survey. Vanna loading controls at 5, 10, and 20 μ L corresponding to 0.5 X, 1.0 X, and 2.0 X respectively. Control lanes Vanna; soft type, McNeal; PINA null, and Canadian Red; PINB null.

PINAa MKALFLIGLLALVASTAFAQYSEVVGSYDVAGGGGAQQCPVETKLNSCRNYLLDRCSTMK
 PINAc MKALFLIGLLALVASTAFAQYSEVVGSYDVAGGGGAQQCPVETKLNSCRNYLLDRCSTMK

 PINAa DFPVTWRWWKWWKGGCQELLGECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASK
 PINAc DFPVTWRWWKWWKGGCQELLGECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASK

 PINAa VIQEAKNLPPRCNQGPPCNIPGTIGYYW
 PINAc VIQEAKNLPPRCNQGPPCNIPGTIGYYW

Figure 8. PINA amino acid sequence comparison between wild-type, Elite 22 PINAa and Elite 14 PINAc. Amino acid substitutions from wild-type are in shaded boxes.

PINBa MKTLFLLALLALVASTTFAQYSEVGGWYNEVGGGGGSQQCPQERPKLSSCKDYVME RCFT
 PINBh MKTLFLLALLALVASTTFAQYSEVGGWYNEVGAAGGSQQCPLEPRLSSCKDYVMGWCFT
 PINBj MKTLFLLALLALVASTTFAQYSEVGGWYNEVGGGGGSQQCPQERPKLRSCKDYVMEWCFT

 PINBa MKDFPVTWPTKWWKGGCEHEVREKCKQLSQIAPQCRCDIRSIR RVIQGRLLGGFLGIWRGEV
 PINBh MKDFPF TWPTKWWKGGCEHEVRENCKQLSQIAPQCRCDIRSIRGMIQGKLGFFGIWRGDV
 PINBj MKDFPVTWPTKWWKGGCEHEVREKCKQLSQIAPQCRCDIRSIRGMIQGKLGFFGIWRGDV

 PINBa FKQLQRAQSLPSKCNMGADCKFPSGYYW
 PINBh FKIQRAQSLPSKCNMGADCKFPSGYYW
 PINBj FKIQRAQSLPSKCNMGADCKFPSGYYW

Figure 9. PINB amino acid sequence comparison between wild-type PINBa, Elite 14 PINBh, and Elite 22 PINBj. Amino acid substitutions from wild-type are in shaded boxes.

To confirm the allelic identity of the elite synthetic parents, *Pina* and *Pinb* coding regions were sequenced using primers described by Gautier et al. (1994). Codominant CAPS markers were developed. Using Biology Workbench (Subramaniam 1998) comparisons were made between *Pinb-D1a* and *Pinb-D1i* and between *Pinb-D1a* and *Pinb-D1j* to identify sequence polymorphisms. The sequence polymorphisms that were identified were searched for restriction endonuclease recognition sites that exploit the sequence variation between the novel alleles as the wild-type allele. The enzyme *Bst*NI recognized one cut site on the *Pinb-D1a* sequence and recognized not cut site on the *Pinb-D1i* sequence. The enzyme *Hpa*II recognized one cut site on the *Pinb-D1a* sequence and recognized not cut site on the *Pinb-Dj* sequence. PCR was performed on parent and F₂ genomic DNA using the primer pair PB5/PB3, the 447bp amplified product was digested with *Bst*NI for Vanna x Elite14 lines and *Hpa*II for Vanna x Elite22 lines. The presence of the wild-type *Pinb-D1a* allelic product digested with *Bst*NI is indicated by 2 bands of sizes 202 and 245 bp (Fig. 10). The presence of the *Pinb-D1i* allelic product digested with *Bst*NI is indicated by a single uncut 447 bp band. The heterozygous *Pinb-D1a / Pinb-D1i* allelic product digested with *Bst*NI is indicated by the presence of all 3 bands (202, 245, and 447 bp). The presence of the wild-type *Pinb-D1a* allelic product digested with *Hpa*II is indicated by 2 bands of lengths 143 and 304 bp (Fig. 10). The presence of the *Pinb-D1j* digested with *Hpa*II is indicated by a single uncut 447 bp band. The heterozygous *Pinb-D1a / Pinb-D1j* allelic product digested with *Hpa*II is indicated by the presence of all 3 bands (143, 304 and 447 bp).

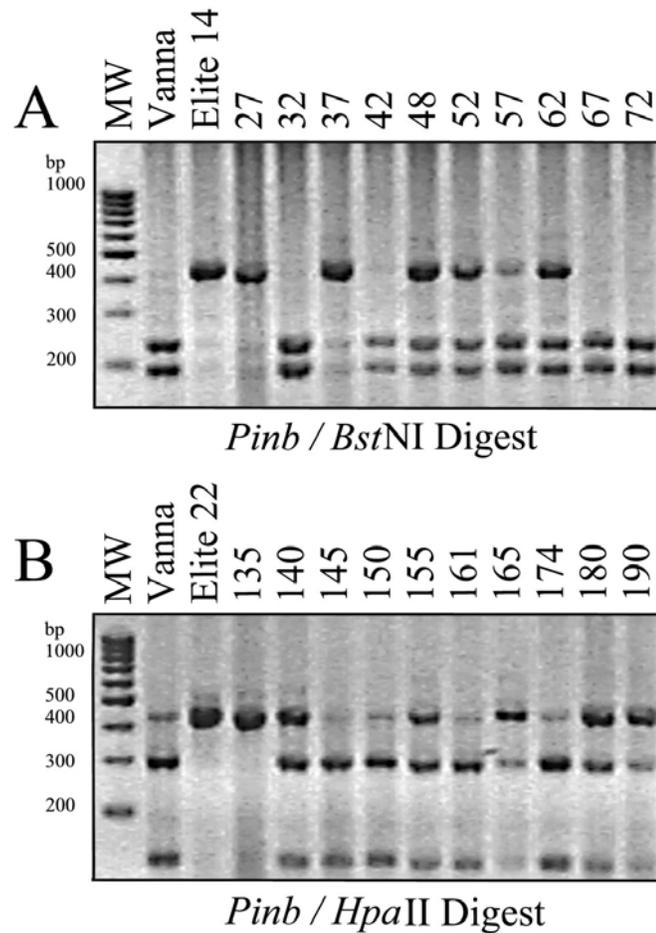


Figure 10. Genotyping subset of $F_{2.4}$ segregating populations and parents via CAPS markers. A) Vanna x Elite14 population (27-72) and parents ‘Vanna’ and Elite 14 parent. The 447 bp *Pinb* PCR products were incubated with restriction endonuclease *Bst*NI. ‘Vanna’ represents the *Pinb-D1a* homozygous class with 1 *Bst*NI restriction site yielding 2 bands of sizes 202 and 245 bp. Elite 14 parent represents the *Pinb-D1i* homozygous class with no *Bst*NI restriction sites yielding an uncut 447 bp band. Lane 48 represents the *Pinb-D1a* / *Pinb-D1i* heterozygous class with all 3 bands (202, 245, and 447 bp). B) Vanna x Elite22 population (135-190) and parents ‘Vanna’ and Elite 22 parent. The 447 bp *Pinb* PCR products were incubated with restriction endonuclease *Hpa*II. ‘Vanna’ represents the *Pinb-D1a* homozygous class with 1 *Hpa*II restriction site yielding 2 bands of sizes 143 and 304 bp. Elite 22 parent represents the *Pinb-D1i* homozygous class with no *Hpa*II restriction sites yielding an uncut 447 bp band. Lane 140 represents the *Pinb-D1a* / *Pinb-D1j* heterozygous class with all 3 bands (143, 304, and 447 bp).

Agronomic yield was measured in the field season of 2006 and revealed no significant differences between *Ha* locus classes in either the Vanna x Elite14 or Vanna x Elite22 segregating population (Table 5). Grain hardness was collected using $F_{2:3}$ seed from the 2005 field season and from the $F_{2:4}$ seed from the 2006 field season. The Vanna x Elite14 segregating population did not display significant differences between *Ha* locus classes. On the other hand, the Vanna x Elite22 segregating population displayed significant grain hardness differences between *Ha* classes as the *Pina-D1a / Pinb-D1j Ha* locus lines had mean grain hardness 7.2 SKCS units higher than the *Pina-D1a / Pinb-D1a Ha* locus lines in 2005 and mean grain hardness 9.6 SKCS units higher than the *Pina-D1a / Pinb-D1a Ha* locus lines in 2006. In 2006 grain hardness was collected on parent types demonstrating ‘Vanna’ was softer than the Elite 22 parent.

Parents ^a	<i>Ha</i> Locus ^d	<i>n</i> (2005)	<i>n</i> (2006)	Agronomic Yield (g) ^e	Grain Hardness (2005) ^f	Grain Hardness (2006) ^g
CS Parent	<i>Pina-D1a/Pinb-D1a</i>		8	189 (23)		45.5 (4.2)
Vanna Parent	<i>Pina-D1a/Pinb-D1a</i>		6	244 (35)		26.7 (1.6)
Elite 22 Parent	<i>Pina-D1-c/Pinb-D1j</i>		2	58 (27)		43.9 (1.3)
Population ^b						
Vanna x Elite 14						
Elite 14	<i>Pina-D1e/Pinb-D1i</i>	17	23	183 (9)	23.4 (1.8)	24.7 (0.9)
Vanna 14	<i>Pina-D1a/Pinb-D1a</i>	31	24	206 (9)	23.1 (2.37)	22.0 (0.9)
<i>P</i>				0.16	0.5	0.18
Population ^c						
Vanna x Elite 22						
Elite 22	<i>Pina-D1a/Pinb-D1j</i>	23	19	147 (13)	32.4 (1.9)	30.7 (0.8)
Vanna 22	<i>Pina-D1a/Pinb-D1a</i>	26	19	141 (9)	25.2 (2.65)	21.2 (1.1)
<i>P</i>				0.35	0.01	<0.001

Table 5. Novel *Ha* loci Effects Upon Yield and Grain Hardness.

^a Comparative statistics of F₂:F₄ seed in 2005 and F₂:F₅ seed in 2006 including parent lines (Elite 14 parent is not represented as there was inadequate yield produced in field).

^b Population means from Vanna x Elite14 cross; Elite 14 represents *Pina-D1e / Pinb-D1i Ha* locus class; Vanna 14 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^c Population means from Vanna x Elite22 cross; Elite 22 represents *Pina-D1a / Pinb-D1j Ha* locus class; Vanna 22 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^d *Pina* and *Pinb* allelic identity for each class in segregating populations for crosses Vanna x Elite14 and Vanna x Elite22 and parents.

^e Total agronomic yield means (standard error) for *Ha* locus class and parents, measured on seed from field 2006 field season.

^f SKCS grain hardness (standard error) for each *Ha* locus class as measured by Perten SKCS 4100.

^g SKCS grain hardness (standard error) for each *Ha* locus class as measured by Perten SKCS 4100.

The *Ha* locus classes in the Vanna x Elite14 segregating population had significant differences in kernel weight and diameter (Table 6) with the *Pina-D1e / Pinb-D1i Ha* locus lines exhibiting a 1.95 mg reduction in mean kernel weight compared to the *Pina-D1a / Pinb-D1a Ha* locus lines and a 0.05 mm reduction in kernel diameter compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. In the Vanna x Elite22 population the *Pina-D1a / Pinb-D1j Ha* locus lines exhibited a 1.39 mg reduction in mean kernel weight compared to the *Pina-D1a / Pinb-D1a Ha* locus lines and a 0.03 mm reduction in kernel diameter. ‘Vanna’ had a heavier but smaller diameter average seed compared to the Elite 22 parent. Grain protein content measurements revealed no significant differences between *Ha* locus classes in the Vanna x Elite14 population. However, in the Vanna x Elite22 population the *Pina-D1a / Pinb-D1j Ha* locus lines exhibited a mean 0.28 % increase in grain protein compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. The parent types displayed mean protein content values with an increase in Elite 22 parent compared the ‘Vanna’.

Parents ^a	<i>Ha</i> Locus ^d	Kernel Weight (mg) ^e	Kernel Diameter (mm) ^f	Protein Content (%) ^g
CS Parent	<i>Pina-D1a/Pinb-D1a</i>	29.3 (3.3)	2.5 (0.10)	15.9 (0.1)
Vanna Parent	<i>Pina-D1a/Pinb-D1a</i>	29.9 (0.6)	2.4 (0.02)	13.1 (0.4)
Elite 22 Parent	<i>Pina-D1a/Pinb-D1j</i>	44.7 (0.4)	2.9 (0.01)	17.1 (0.7)
Population ^b				
Vanna x Elite 14				
Elite 14	<i>Pina-D1e/Pinb-D1i</i>	37.3 (0.6)	2.6 (0.02)	14.4 (0.1)
Vanna 14	<i>Pina-D1a/Pinb-D1a</i>	39.3 (0.5)	2.7 (0.02)	14.3 (0.1)
<i>P</i>		0.01	0.01	0.3
Population ^c				
Vanna x Elite 22				
Elite 22	<i>Pina-D1a/Pinb-D1j</i>	39.3 (0.4)	2.7 (0.01)	14.5 (0.1)
Vanna 22	<i>Pina-D1a/Pinb-D1a</i>	40.7 (0.5)	2.8 (0.02)	14.8 (0.1)
<i>P</i>		0.01	0.07	0.05

Table 6. Novel *Ha* loci Effects Upon Kernel Traits.

^a Comparative statistics of F₂:F₄ seed in 2005 and F₂:F₅ seed in 2006 including parent lines (Elite 14 parent is not represented as there was inadequate yield produced in field).

^b Population means from Vanna x Elite14 cross; Elite 14 represents *Pina-D1e / Pinb-D1i Ha* locus class; Vanna 14 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^c Population means from Vanna x Elite22 cross; Elite 22 represents *Pina-D1a / Pinb-D1j Ha* locus class; Vanna 22 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^d *Pina* and *Pinb* allelic identity for each class in segregating populations form crosses Vanna x Elite14 and Vanna x Elite22 and parents.

^e Kernel weight means (standard error) for each *Ha* locus class and parents as grown in 2006 as measured by Perten SKCS 4100 (mg).

^f Kernel diameter means (standard error) for each *Ha* locus class and parents grown in 2006 as measured by Perten SKCS (mm).

^g Kernel protein content means (standard error) for each homozygous class and parents grown in 2006 as measured NIR (%).

Milling was carried out on 50 g samples of F_{2:4} and parent seed pools using a Quadrumat Brabender Jr. mill. The milling and flour traits measured showed significant differences between classes in both populations for most traits (Table 7). Among the Vanna x Elite14 population, *Pina-D1e / Pinb-D1i Ha* locus lines exhibited a 0.68 % increase in flour yield compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. Among the Vanna x Elite22 population there was variation as the *Pina-D1a / Pinb-D1j Ha* locus lines exhibited a 2.15 % increase in mean flour yield compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. Among the parent entries, Elite 22 had the greatest flour yield followed by ‘Vanna’. The *Pina-D1e / Pinb-D1i Ha* locus lines displayed flour ash content values 0.02 % higher than the *Pina-D1a / Pinb-D1a Ha* locus lines. The *Pina-D1a / Pinb-D1j Ha* locus lines displayed flour ash values 0.02 % higher than the *Pina-D1a / Pinb-D1a Ha* locus lines. Ash content for parents ranged from 0.51 % for ‘Vanna’ to 0.69 % for Elite 22. Starch damage analysis performed on flour from *Pina-D1e / Pinb-D1i Ha* locus lines had a 0.37 % increase in starch damage compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. In the Vanna x Elite22 population there was no significant difference in starch damage between classes. The parents had mean starch damage values ranging from 2.91 % for ‘Vanna’ to 2.79 % for Elite 22. Protein content measurements on flour showed *Pina-D1e / Pinb-D1i Ha* locus lines had values 0.4 % higher than the *Pina-D1a / Pinb-D1a Ha* locus lines. Further, the flour protein content from *Pina-D1a / Pinb-D1j Ha* locus lines were 0.51 % lower than the *Pina-D1a / Pinb-D1a Ha* locus lines. The parents differed markedly in flour protein content from 12.08 % for ‘Vanna’ to 17.17 % for Elite 22.

Parents ^a	<i>Ha</i> Locus ^d	%Flour Yield ^e	% Flour Ash ^f	% Starch Damage ^g	Flour Protein (%) ^h
CS Parent	<i>Pina-D1a/Pinb-D1a</i>	69.8 (0.9)	0.56 (0.32)	2.81 (0.28)	15.9 (0.1)
Vanna Parent	<i>Pina-D1a/Pinb-D1a</i>	71.6 (0.5)	0.51 (0.10)	2.20 (0.19)	12.1 (0.7)
Elite 22 Parent	<i>Pina-D1a/Pinb-D1j</i>	72.9 (1.9)	0.69 (0.01)	2.79 (0.42)	17.2 (0.4)
Population ^b					
Vanna x Elite 14					
Elite 14	<i>Pina-D1e/Pinb-D1i</i>	70.4 (0.3)	0.53 (0.01)	2.61 (0.12)	13.8 (0.2)
Vanna 14	<i>Pina-D1a/Pinb-D1a</i>	69.8 (0.3)	0.51 (0.00)	2.24 (0.13)	13.4 (0.1)
<i>P</i>		0.06	0.002	0.02	0.05
Population ^c					
Vanna x Elite 22					
Elite 22	<i>Pina-D1a/Pinb-D1j</i>	70.4 (0.4)	0.54 (0.01)	2.59 (0.13)	13.7 (0.2)
Vanna 22	<i>Pina-D1a/Pinb-D1a</i>	68.2 (0.5)	0.52 (0.00)	2.55 (0.12)	14.2 (0.2)
<i>P</i>		0.001	0.04	0.41	0.04

Table 7. Novel *Ha* loci Effects Upon Milling Traits.

^a Comparative statistics of F₂:F₄ seed in 2005 and F₂:F₅ seed in 2006 including parent lines (Elite 14 parent is not represented as there was inadequate yield produced in field).

^b Population means from Vanna x Elite14 cross; Elite 14 represents *Pina-D1e / Pinb-D1i Ha* locus class; Vanna 14 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^c Population means from Vanna x Elite22 cross; Elite 22 represents *Pina-D1a / Pinb-D1j Ha* locus class; Vanna 22 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^d *Pina* and *Pinb* allelic identity for each class in segregating populations from crosses Vanna x Elite14 and Vanna x Elite22 and parents.

^e Total flour yield as a percentage of total milling products recovered means (standard error) for each *Ha* locus class and parents milled on Brabender Quad Jr. mill.

^f Percentage of flour ash means (standard error) for each *Ha* locus class and parents as measured by weight before and after incantation (%).

^g Percentage of flour damaged starch means (standard error) for *Ha* locus class and parents as measured by Megazyme kit (%).

^h Flour protein means (standard error) for each *Ha* locus class and parents as measured by NIR (%).

Flour from *Ha* locus classes and parents was sifted to determine flour particle size distribution (Table 8). The significant differences in particle size distribution in both the Vanna x Elite14 and Vanna x Elite22 segregating populations are most evident in the smallest particle size range of <0.053 mm. The percent flour <0.053 mm in the *Pina-D1e / Pinb-D1i Ha* locus lines was 2.28 % lower than the *Pina-D1a / Pinb-D1a Ha* locus lines. Though not statistically significant there was an increase in all other size ranges for composition for the *Pina-D1e / Pinb-D1i Ha* locus lines compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. In the Vanna x Elite22 population also exhibited significant differences in percent of total recovered flour in the particle size range of <0.053 mm with the *Pina-D1a / Pinb-D1j Ha* locus mean values 1.94 % lower than the *Pina-D1a / Pinb-D1a Ha* locus means. Though not statistically significant there was a decrease in percent recovery for the next smallest size range of 0.074-0.053 mm and an increase in the largest particle size range for the *Pina-D1a / Pinb-D1j Ha* locus lines compared to the *Pina-D1a / Pinb-D1a Ha* locus lines. The ‘Vanna’ parent displayed a more than threefold increase in percent of total recovered flour in the particle size range of <0.053 mm compared to the Elite 22 parent.

		% Total Recovery				
		Size of Flour Particles (mm) ^e				
Parents ^a	<i>Ha</i> Locus ^d	>0.300	0.300-0.149	0.149-0.074	0.074-0.053	<0.053
CS Parent	<i>Pina-D1a/Pinb-D1a</i>	0.000	27.26 (0.54)	42.07 (0.53)	19.56 (0.61)	11.11 (0.51)
Vanna Parent	<i>Pina-D1a/Pinb-D1a</i>	0.000	19.47 (0.56)	22.11 (0.48)	27.37 (0.51)	31.05 (0.51)
Elite 22 Parent	<i>Pina-D1a/Pinb-D1j</i>	0.000	30.10 (1.53)	46.43 (0.51)	14.80 (0.51)	8.67 (0.53)
Population ^b						
Vanna x Elite 14						
Elite 14	<i>Pina-D1e/Pinb-D1i</i>	0.18 (0.06)	23.46 (0.45)	29.80 (1.79)	22.99 (1.66)	23.66 (0.63)
Vanna 14	<i>Pina-D1a/Pinb-D1a</i>	0.17 (0.05)	23.04 (0.44)	28.72 (1.43)	22.34 (1.40)	25.94 (0.71)
<i>P</i>		0.41	0.25	0.32	0.38	0.01
Population ^c						
Vanna x Elite 22						
Elite 22	<i>Pina-D1a/Pinb-D1j</i>	0.000	24.68 (0.43)	33.60 (1.64)	22.77 (1.12)	18.94 (0.62)
Vanna 22	<i>Pina-D1a/Pinb-D1a</i>	0.000	24.60 (0.45)	30.60 (1.71)	23.92 (1.10)	20.88 (0.90)
<i>P</i>			0.45	0.11	0.23	0.04

Table 8. Novel *Ha* loci Effects on Flour Particle Size Composition.

^a Comparative statistics of F₂:F₄ seed in 2005 and F₂:F₅ seed in 2006 including parent lines (Elite 14 parent is not represented as there was inadequate yield produced in field).

^b Population means from Vanna x Elite14 cross; Elite 14 represents *Pina-D1e / Pinb-D1i Ha* locus class; Vanna 14 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^c Population means from Vanna x Elite22 cross; Elite 22 represents *Pina-D1a / Pinb-D1j Ha* locus class; Vanna 22 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^d *Pina* and *Pinb* allelic identity for each class in segregating populations form crosses Vanna x Elite14 and Vanna x Elite22 and parents.

^e Percentage of total flour product recovered in each size range given in (mm).

In order to determine the linearity of PIN ionization on MALDI-TOF, of PIN as compared to the standard protein Cytochrome *c* standard curves of PIN and Cytochrome *c* were produced based on replicated spectra obtained for each protein independently (Fig. 11). The standard curve of total PIN (Fig. 11 A) and of Cytochrome *c* (Fig. 11 B) shows that relative intensity is directly proportional to the concentration. Mean spectra obtained from total PIN preparations revealed an increase in PIN levels in synthetic derived *Ha* locus in both populations. MALDI-TOF comparative quantification of Vanna x Elite14 population indicated a 15.6 % increase in total PIN among *Pina-D1e / Pinb-D1i Ha* locus lines compared to *Pina-D1a / Pinb-D1a Ha* locus lines (Table 9). In the Vanna x Elite22 population a 23.1 % increase in total PIN among *Pina-D1a / Pinb-D1j Ha* locus lines compared to *Pina-D1a / Pinb-D1a Ha* locus lines was observed. The mean values are averaged spectra from four randomly chosen lines with one representative a spectrum from each class is shown in Fig. 12. This quantification showed an approximate doubling of total PIN levels in the Elite 22 parent compared to ‘Vanna’. The MALDI-TOF data were complemented by SDS-PAGE (Fig. 13) images, prepared using the same TX-114 samples as were used for MALDI-TOF, indicating increased levels of total PIN in synthetic *Ha* locus lines in both Vanna x Elite14 and Vanna x Elite22 segregating populations.

Parents ^a	<i>Ha</i> Locus ^d	Calculated Protein (pmol PIN / mg total protein) ^e
Vanna Parent	<i>Pina-D1a/Pinb-D1a</i>	13.7 (0.3)
Elite 22 Parent	<i>Pina-D1-c/Pinb-D1j</i>	29.4 (0.6)
Population ^b		
Vanna x Elite 14		
Elite 14	<i>Pina-D1e/Pinb-D1i</i>	33.4 (0.6)
Vanna 14	<i>Pina-D1a/Pinb-D1a</i>	28.2 (0.2)
<i>P</i>		0.0001
Population ^c		
Vanna x Elite 22		
Elite 22	<i>Pina-D1-c/Pinb-D1j</i>	37.1 (0.5)
Vanna 22	<i>Pina-D1a/Pinb-D1a</i>	28.6 (0.3)
<i>P</i>		<0.0001

Table 9. MALDI-TOF Relative Qualification of Total PIN from TX-114 Fractionation.

^a Comparative statistics of F₂:F₄ seed in 2005 and F₂:F₅ seed in 2006 including parent lines (Elite 14 parent is not represented as there was inadequate yield produced in field).

^b Population means from Vanna x Elite14 cross; Elite 14 represents *Pina-D1e / Pinb-D1i Ha* locus class; Vanna 14 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^c Population means from Vanna x Elite22 cross; Elite 22 represents *Pina-D1a / Pinb-D1j Ha* locus class; Vanna 22 represents *Pina-D1a / Pinb-D1a Ha* locus class.

^d *Pina* and *Pinb* allelic identity for each class in segregating populations from crosses Vanna x Elite14 and Vanna x Elite22 and parents.

^e Calculated total PIN protein content per weight of total starting flour (standard error) averaged across four randomly chosen lines in each *Ha* locus class and parents (pmol/mg) as fit to Cytochrome *c* standard curve.

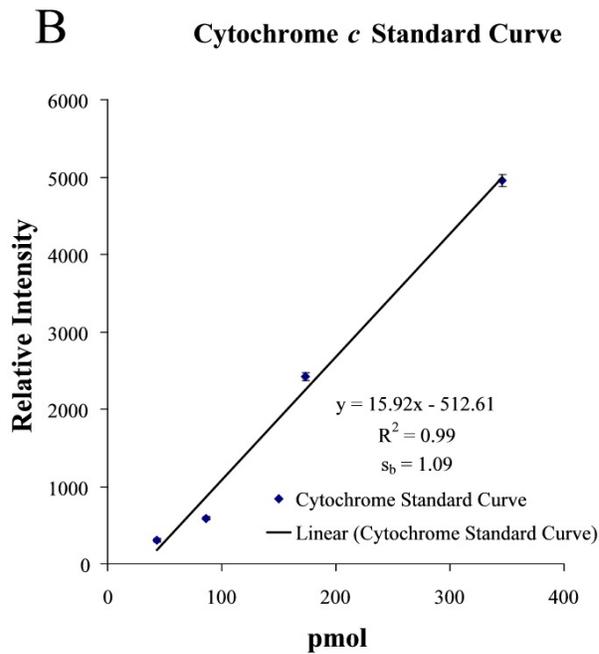
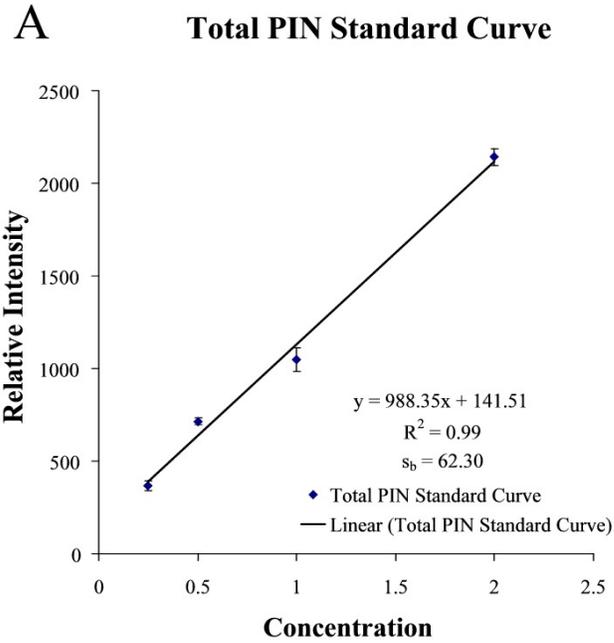


Figure 11. Standard curve from MALDI-TOF produced using loading controls ranging in concentration values from 0.25 X to 2.0 X and corresponding intensity given in relative units for A. Total PIN from soft wheat Heron TX-114 preparation. B. Pure Cytochrome *c* standard at 173 pmol/ μ L suspended in water.

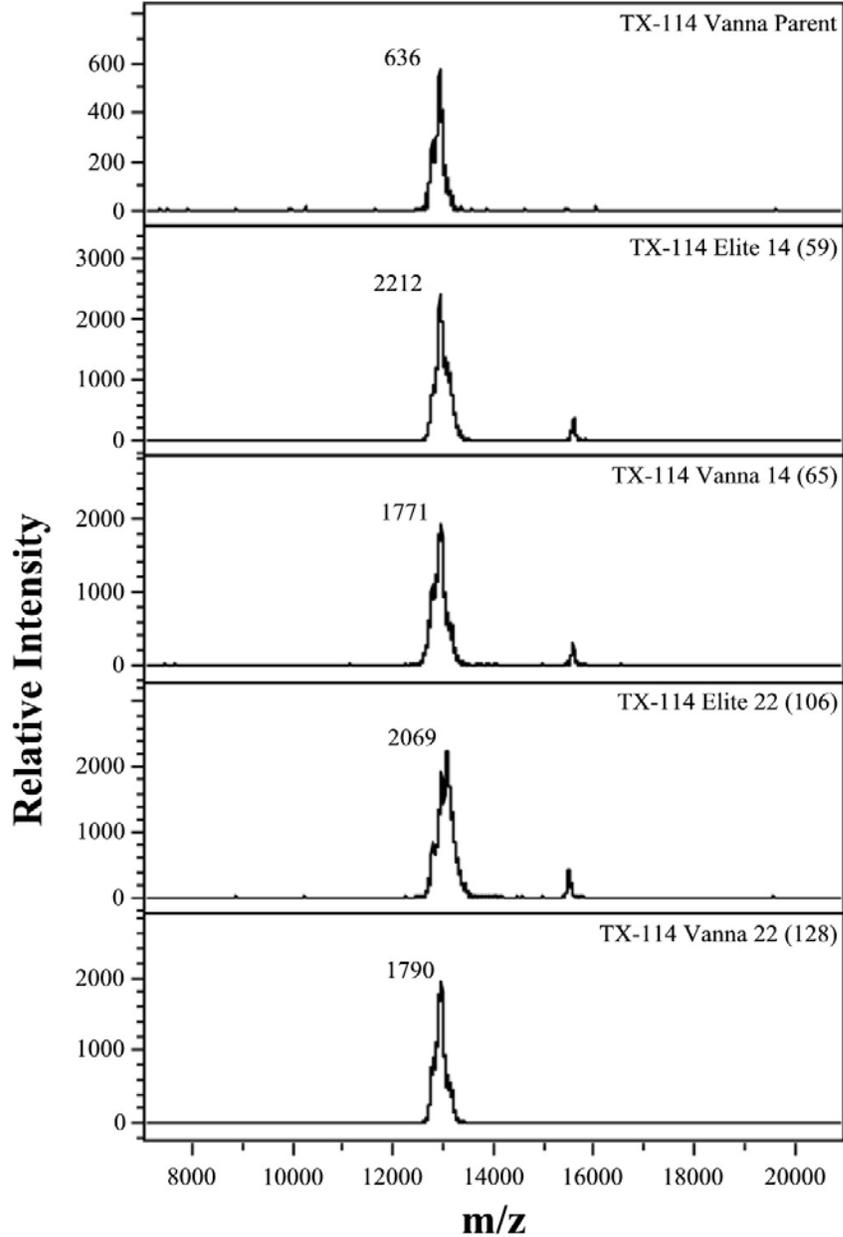


Figure 12. MALDI-TOF Representative Spectra. A spectrum from one of four randomly chosen lines in each *Ha* locus class and parent lines. Vanna parent represents spectra from 'Vanna' parent. Elite 14 (59) represents *Pina-D1e / Pinb-D1i Ha* locus class; Vanna 14(65) represents *Pina-D1a / Pinb-D1a Ha* locus class. Elite 22 (106) represents *Pina-D1-c/Pinb-D1j Ha* locus class; Vanna 22 (128) represent *Pina-D1a/Pinb-D1a Ha* locus class. The exact intensity is given next to PIN peak given in relative units at 13,000 m/z.

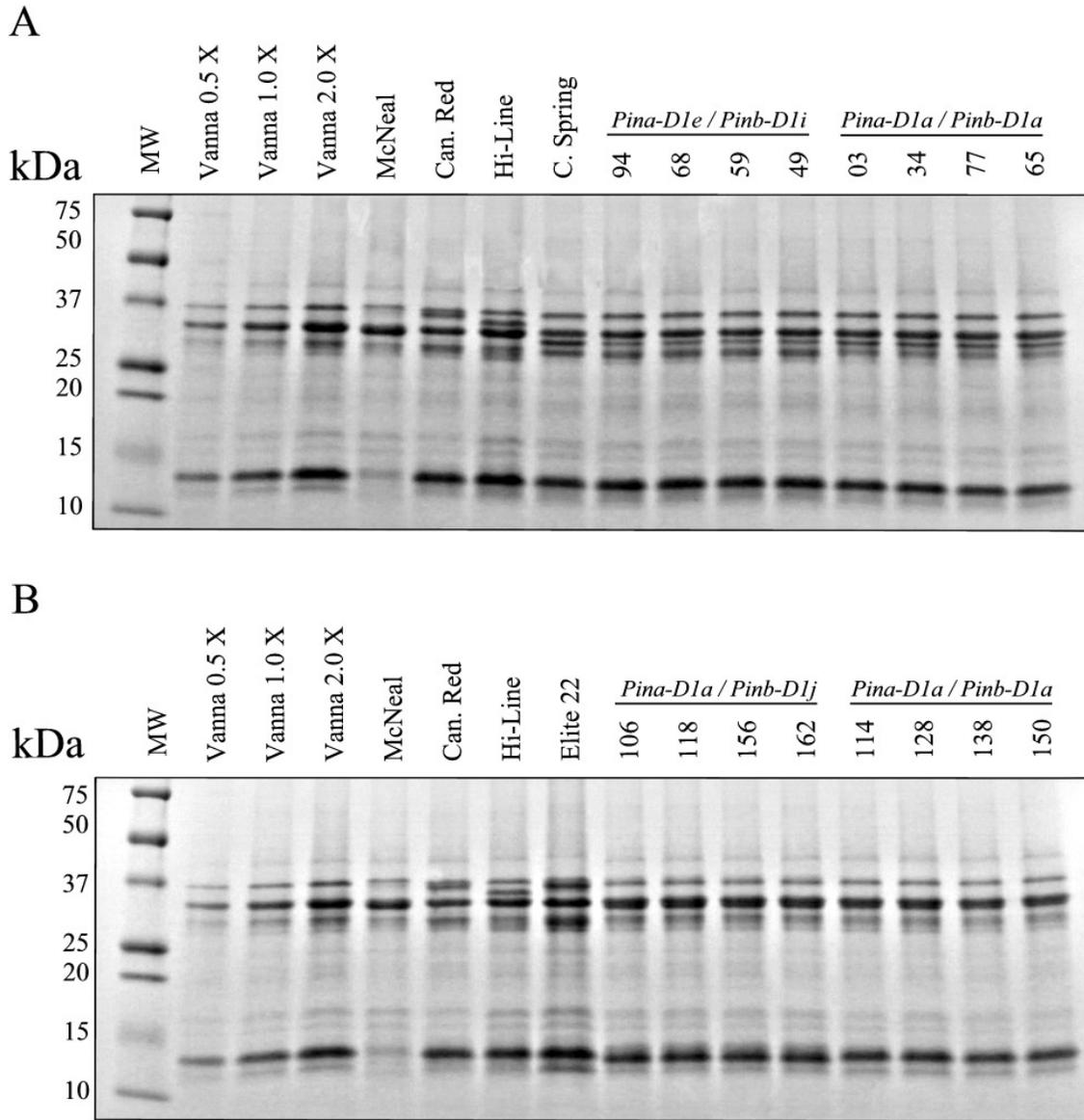


Figure 13. SDS-PAGE Gel Fragmentation of Total PIN. A) Total PIN fractions from TX-114 extraction of Vanna x Elite 14 $F_{2.5}$ *Ha* locus classes. B) Total PIN fractions from TX-114 extraction in Vanna x Elite $F_{2.5}$ *Ha* locus classes. Soft wheat loading control ‘Vanna’ at 3, 6, and 12 μ L representing 0.5 X, 1.0 X and 2.0 X concentrations respectively, McNeal; PINA null, and Canadian Red; PINB null. Lanes 94, 68, 59, and 49 represent the *Pina-D1e / Pinb-D1i* *Ha* locus class. Lanes 03, 34, 77, and 65 represent the *Pina-D1a / Pinb-D1a* *Ha* locus class. Lanes 106, 118, 156, and 162 represent the *Pina-D1a / Pinb-D1j* *Ha* locus class. Lanes 114, 128, 138, and 150 represent the *Pina-D1a / Pinb-D1a* *Ha* locus class.

Discussion

Kernel texture is a primary determinant of end product quality in commercial hexaploid wheat. All traded hexaploid wheats are categorized as either “hard” or “soft” based on grain hardness. This fundamentally important quality is associated with many end product traits that results in hard wheat being best for bread baking and soft wheat being best for baking pastry goods (Reviewed in Morris and Rose 1996). Hard wheats exhibit increased flour yield, increased starch damage, increased protein content, increased flour ash content, and larger mean particle size compared to soft wheats (Symes 1969; Rogers et al. 1993).

Variation in grain hardness is predominantly governed by the *Ha* locus, containing the *Pina* and *Pinb* genes (Jolly et al. 1993; Sourdille et al. 1996; Giroux and Morris 1998). In hexaploid wheat (AABBDD) the only functional copies of the *Pin* genes are found on the D genome. Although *Gsp* is contained within the *Ha* locus, only the *Pin* genes have been functionally associated with grain hardness (Tranquilli et al. 1999; Gautier et al. 2000; Chantret et al. 2005). All surveyed hexaploid soft wheats carry the same *Pin* alleles, *Pina-D1a* and *Pinb-D1a*. Any allelic change in these genes results in a hard phenotype (Giroux and Morris 1997; 1998). Grain texture is secondarily governed by many minor genes (Reviewed in Anjum and Walker 1991).

The causative relationship between *Puroindoline* mutations and hard grain texture was confirmed through studies where soft phenotypes were recovered when hard textured genotypes were complemented with wild-type *Pin* alleles (Beecher et al. 2002; Martin et

al. 2006). The starch surface associated protein complex friabilin is comprised of PINA and PINB. This complex is present at higher levels in soft wheats compared to hard wheats (Greenwell and Schofield 1986). Reduction in grain hardness in soft wheats has been obtained by overexpression of the wild-type *Pin* alleles in a soft wheat genetic background (See et al. 2004; Campbell et al. 2007).

A significant number of *Puroindoline* sequence polymorphisms have been identified in diploid *Ae. tauschii* and synthetic hexaploid wheat lines (Massa et al. 2004; Gedye et al. 2004; Chen et al. 2005). Previously published literature indicated that the PIN protein isoforms PINAc, and PINBh would have observable effects on grain texture (Gedye et al. 2004). Published data collected showed synthetic seed expressing PINAc had a 9.3 SKCS unit reduction compared to synthetic seed expressing PINAa and seed expressing PINBh had a 4.6 SKCS unit reduction compared to synthetic seed expressing PINBa (Gedye et al. 2004). The observed grain texture differences may be confounded by other linked factors, since the genotypes surveyed did not represent a defined family structure. The translated proteins PINBh and PINBj share eight amino acid substitutions, PINBj has only one unique amino acid substitution versus PINBh which consists of a serine (uncharged polar) to arginine (charged polar) at position 19. The translated protein PINAc has one amino acid substitution that distinguishes it from PINAa, at position 58 an arginine (charged polar) is changed to a glutamine (uncharged polar). The translated protein PINBh has a total of thirteen amino acid substitutions relative to protein PINBa. The translated PINBj has a

total of nine amino acid substitutions versus PINBa. The *Ha* loci chosen for investigation here allow for the elucidation of the allelic effects of *Pina-D1e* / *Pinb-D1i* while having the capability of simultaneously investigating the effects of a synthetic background effect using the assumed negative control *Ha* locus *Pina-D1a* / *Pinb-D1j*.

We demonstrated that the alleles *Pina-D1a* / *Pinb-D1j* expressed together do not significantly affect grain hardness and that the alleles *Pina-D1e* / *Pinb-D1i* expressed together significantly increase grain hardness. Further, there are observed changes in kernel, flour and milling quality traits in a direction that is of potential use in improving end product quality. The kernel traits associated with the increased grain hardness are kernel weight, kernel diameter, and protein content. The flour and milling traits associated with the increased grain hardness include flour yield, flour ash content, flour protein, and flour particle size composition. The *Pina-D1e* / *Pinb-D1i* *Ha* locus lines had unchanged agronomic yield, unchanged grain hardness, reduced kernel weight, reduced kernel diameter, unchanged kernel protein content, and increased levels of total PIN compared to *Pina-D1a* / *Pinb-D1a* *Ha* locus lines. The *Pina-D1a* / *Pinb-D1j* *Ha* locus lines had unchanged agronomic yield, increased grain hardness, reduced kernel weight, reduced kernel diameter, decreased kernel protein content, and increased levels of total PIN compared to *Pina-D1a* / *Pinb-D1a* *Ha* locus line. Flour produced from small scale milling of *Pina-D1e* / *Pinb-D1i* *Ha* locus lines had increased flour yield, increased flour ash, increased starch damage, increased flour protein content and a larger mean particle size compared to *Pina-D1a* / *Pinb-D1a* *Ha* locus lines.

Flour produced from small scale milling of *Pina-D1a / Pinb-D1j Ha* locus lines had increased flour yield, increased flour ash, unchanged starch damage, decreased flour protein content and a larger mean particle size compared to *Pina-D1a / Pinb-D1a Ha* locus lines.

Here we present MALDI-TOF as an alternative to SDS-PAGE for evaluating protein levels. While visually approximating protein levels from gels is rapid and informative, it is subjective and may not allow for determination of small differences. MALDI-TOF allows for the quantitative measurement of small differences in PIN protein concentrations. Though MALDI-TOF does not exhibit the resolving power of some other mass spectrometry techniques it is a repeatable method for determining relative levels of proteins (Bothner et al. 1999) as is demonstrated by the excellent fit $R^2 = 0.99$ to the linear regression obtained from loading controls of PIN and a pure protein within the same mass range as PIN (Cytochrome *c*). The observed increase in PIN levels associated with both introgressed synthetic *Ha* loci is unexpected as these lines also demonstrate increased SKCS grain hardness. Taken in concert, the data indicate that the observed increase in kernel texture in both populations maybe the result of other genetic factors and not solely PIN expression. It should be noted that the analyzed lines were not isolines but $F_{2:4}$ and $F_{2:5}$, as such substantial genetic background effects are anticipated. It remains to be seen if the allelic effects observed here will be observed in recombinant inbred lines, or if the isolation of these alleles from the synthetic background will produce an independent set of characteristics.

Conclusion

The data presented here demonstrates that *Pina-D1e / Pinb-D1i Ha* locus lines and *Pina-D1a/ Pinb-D1j Ha* locus lines had distinctive sets of kernel, flour and milling properties. *Pina-D1e / Pinb-D1i Ha* locus lines showed reduced kernel weight, reduced kernel diameter, and increased flour ash, increased starch damage, increased flour protein content, increased PIN levels, and a larger mean particle size. The *Pina-D1a/ Pinb-D1j Ha* locus lines showed unchanged agronomic yield, increased grain hardness, reduced kernel weight, reduced kernel diameter, decreased kernel protein content, increased flour yield, increased flour ash, unchanged starch damage, decreased flour protein content, increased PIN levels, and a larger mean particle size.

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