INCREASED WHEAT (*TRITICUM AESTIVUM* L.) GRAIN HARDNESS CONFERRED BY NOVEL PUROINDOLINE HAPLOTYPES AND ITS EFFECTS ON END USE QUALITY

by

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ABSTRACT

The *Puroindoline* genes (*Pina* and *Pinb*) together comprise the wheat (*Triticum aestivum* L.) *Hardness* locus (*Ha*) located on chromosome 5D and control grain texture. While hard wheats contain a mutation in either *Pina* or *Pinb*, there is no puroindoline allelic diversity among soft hexaploid wheats as all tested soft wheats carry the *Pina-D1a/Pinb-D1a* alleles. However, abundant *Pina* and *Pinb* allelic variation exists within synthetic hexaploid wheats created using novel D genome donors. A previous study indicated that the *Pina-D1c/Pinb-D1h* haplotype conferred a 10 unit decrease in hardness compared to the *Pina-D1a/Pinb-D1a Ha* haplotype. Here we tested the effects of four novel *Ha* locus haplotypes (*Pina-D1c/Pinb-D1h, Pina-D1e/Pinb-D1i, Pina-D1a/Pinb-D1i,* and *Pina-D1j/Pinb-D1i*) from synthetic wheats by crossing *Pina-D1c/Pinb-D1h* into the soft white spring wheat, 'Alpowa' and all four haplotypes into the soft white spring wheat, 'Vanna'. PINAa/PINBh, PINAc/PINBh, and PINAj/PINBh did not affect protein levels and *Pina-D1c/Pinb-D1h* did not affect transcript levels found in the seed. *Pina-D1c/Pinb-D1h* was found to increase grain hardness relative to the wild type *Ha* locus by an average of 6.5 units for all populations, *Pina-D1e/Pinb-D1i* by 5.6 units, *Pina-D1a/Pinb-D1i* by 12.6 units, and *Pina-D1j/Pinb-D1i* by 3.8 units. In examining the effects of one of these novel loci, *Pina-D1c/Pinb-D1h,* on end use quality, it is evident that the synthetic *Ha* haplotype can decrease sugar snap cookie diameter and increase flour particle size. Other baking quality traits appear to be unaffected by the *Pina-D1ac/Pinb-D1h Ha* haplotype. These findings may be beneficial to breeders looking to improve the baking qualities of high yielding soft wheat varieties.

CHAPTER 1

INCREASED WHEAT (*TRITICUM AESTIVUM* L.) GRAIN HARDNESS CONFERRED BY NOVEL PUROINDOLINE HAPLOTYPES FROM *AEGILOPS TAUSCHII*

Introduction

Grain texture is an important factor in the classification of hexaploid (*Triticum aestivum* L.) wheat and helps to determine end use quality (reviewed in Morris and Rose, 1996). The Hardness (*Ha*) locus, the major contributing factor to grain texture, is simply inherited (Symes, 1965) and is located on the short arm of chromosome 5 (Law et. al. 1978) of all three hexaploid wheat diploid progenitor species (Dubcovsky and Dvorak 1995; Jolly et al. 1996; Sourdille et al. 1996; Giroux and Morris 1997). Bread wheat evolved from a hybridization of *Triticum urartu* (AA) and a relative of Aegilops speltoides (BB), to form *Triticum turgidum* ssp. *dicoccoides* (AABB). A subsequent hybridization of *Aegilops tauschii* (DD) and a domesticated tetraploid wheat species, likely *Triticum turgidum* ssp. *dicoccum* (AABB; Reviewed in Feldman et. al. 1995), resulted in common hexaploid wheat (*Triticum aestivum* L. (AABBDD). Interestingly, the *Ha* locus is absent on both the 5A and 5B chromosomes of hexaploid wheat due to a deletion which occurred during the tetraploidization event and independently and recurrently among different polyploid wheats (Li et al. 2008). Therefore, introgression of diverse *Ha* loci would require new D genome donors. *Ha* locus diversity exists among *A. tauschii* accessions (Massa et. al. 2004). By recreating the hybridization of T. turgidum

and *A. tauschii* (Mujeeb-Kazi et. al. 1996), novel *Ha* loci can be incorporated into synthetic wheats and then intercrossed with modern cultivars.

The *Ha* locus contains 3 linked genes, *Puroindoline-a (Pina*) (Sourdille et al. 1996; Giroux and Morris, 1998), *Puroindoline-b* (*Pinb*) (Giroux and Morris, 1997), and *Grain softness protein* (Gsp-1) (Jolly et al. 1996), which are collectively contained within a 70 kbp DNA segment on the short arm of chromosome 5D (Chantret et al. 2005). The starch granule bound marker protein for grain softness, friabilin (Greenwell and Schofield, 1986), is made up of PINA and PINB and there is no direct evidence that GSP-1 has any effect on grain hardness, despite the tight linkage of the three genes (Giroux and Morris, 1998). Surveys of *Pina* and *Pinb* alleles present in hexaploid wheat varieties have found that all soft wheats contain the *Ha* locus haplotype *Pina-D1a/Pinb-D1a*, while all hard wheats contain a point mutation in either *Pina* or *Pinb* (Giroux and Morris, 1998). Pins account for less than 12% of the hardness differences among hard wheats (Giroux et al. 2000), but in crosses between hard and soft wheats, they account for nearly 60% of the differences (Campbell et al. 1999). Diverse *Pina*-D1/*Pinb*-D1 haplotypes that give soft grain phenotype would allow for studies of Pin function, and could provide improved soft wheat milling and baking quality. Novel haplotypes could either be created via mutagenesis of hexaploid wheats (Feiz et al. 2009a) or from the use of novel D genome donors. In fact, 19 unique *Pina*-D1/*Pinb*-D1 haplotypes were found among 50 *A. tauschii* and 75 synthetic hexaploid accessions (Massa et al. 2004; Gedye et al. 2004). Grain hardness was measured on 75 synthetic hexaploids varying for *Ha* haplotype as a result of having different *A. tauschii* donors. Gedye et al. (2004) found two alleles, *Pina*-

D1c and *Pinb-D1h*, independently were associated with a 9.3 and a 4.6 unit decrease in hardness, respectively. However, Gedye et al. (2004) did not examine linkage between the novel *Ha* haplotypes and grain texture and therefore the observed differences could have been due to genes unlinked to *Ha*.

Novel *Ha* haplotypes could also have an impact on milling and baking quality. Hard wheats are generally used for breads and soft wheats are generally used for cakes and cookies (Rogers et al. 1993). The major grain hardness quality associated traits are those affected by changes in starch damage, particle size, and size distribution after milling, which lead to different swelling, pasting, and gelling qualities (Dengate, 1984). Within the hard wheat class, relatively minor (<10 hardness unit) *Ha* locus mediated hardness changes impact nearly all milling and baking traits (Martin et al. 2001, 2008). Within the soft wheat market class, Campbell et al. (2007) found that increasing puroindoline dosage via chromosome substitution reduced grain hardness and modified milling properties. Therefore, functional *Ha* locus haplotypes that alter PINA and/or PINB functionality could modify both grain hardness and milling and baking quality. However, novel *Ha* haplotypes from *A. tauschii* would likely be of value only in modifying soft wheat end product quality as the grain hardness values of all tested synthetic hexaploid wheats are within the soft wheat class (Gedye et. al. 2004)

Here, near isogenic lines were used to further investigate the impact of these novel haplotypes on grain texture while minimizing other background effects. F_2's were then used to test hardness of all four novel haplotypes. The data indicate that all novel *Ha*

haplotypes confer increased grain hardness without significantly affecting the expression of *Pina* or *Pinb* at the transcript or protein level.

Materials and Methods

Plant Material

Nine synthetic wheat accessions were randomly selected from the Kansas State Wheat Genetic Resource Center (Table 1). Genomic DNA was extracted from an individual seedling (Riede and Anderson 1996) of each accession with each sequenced plant being used as the seed source of the novel *Ha* locus in all subsequent experiments. *Pina* and *Pinb* coding sequences were then amplified from each accession using specific primers (Table 2) and directly sequenced (SeqWright, Houston, TX) with the amplification primers following gel purification using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The PCR cycling parameters consisted of an initial denaturation of 95ºC for 3 min, followed by 40 cycles of 95ºC for 30 sec, 50ºC for 30 sec, 72ºC for 1 min, and a final extension at 72ºC for 8 min. Crosses were made between elite synthetic line #14 (Syn14, Table 1) containing the haplotype *Pina-D1c/Pinb-D1h* and the soft white spring wheat varieties 'Alpowa' (PI 566596; 1994 release from Washington State University) and 'Vanna' (PI 587199; 1995 release for Western Plant Breeders Inc.); both of which have the soft wheat *Ha* locus haplotype *Pina-D1a/Pinb-D1a*). 'Alpowa' and 'Vanna' were used as the female parent in the first and all subsequent backcrosses. The 'Alpowa' x Syn14 population was backcrossed three times and the 'Vanna' x Syn14 population was backcrossed two times. Selection for the novel *Ha* haplotype was done in every generation, starting with selection of heterozygotes in the F1's. For the 'Alpowa' x

Syn14 population, *Ha* locus heterozygotes were followed through the BC_3F_1 generation with BC_3F_2's used to determine single gene *Ha* segregation ratios and to select homozygous individuals with either the *Pina-D1c/Pinb-D1h* locus and or the *Pina-D1a/Pinb-D1a* locus. For the 'Vanna' population, *Ha* locus heterozygotes were followed through the BC_2F_1 generation with BC_2F_2's used the same as for the 'Alpowa' population. Selection was done on DNA from individual BC_2F_2 and BC_3F_2 plants, respectively, while PIN expression levels were measured using $BC_3F_{2:3}$ and $BC_2F_{2:3}$ seeds. Eight additional synthetic wheat accessions (Table 1) with five containing the *Pina-D1c/Pinb-D1h* haplotype and one each for the *Pina-D1e/Pinb-D1i*, the *Pina-*D1a/*Pinb*-D1i and the *Pina-D1j/Pinb-D1i* haplotypes were crossed with 'Vanna'. F_2's were used to determine single gene *Ha* segregation ratios and to select homozygous individuals with either the synthetic *Ha* locus and or the *Pina-D1a/Pinb-D1a Ha* locus.

Table 1. Synthetic hexploid parental lines and ID's based on the Kansas State University Wheat Genetics Resource Center database of CIMMYT Elite 97 Synthetic Lines

Elite # [a]	Generation studied	*Ha* locus haplotype	Protein haplotype	Cross ID # [a]	Durum genome donor [a,b]	*A. tauschii* donor accession [a]
7	F_2	*Pina-D1e/Pinb-D1i*	PINAc/PINBh	CIGM87.2770-1B-0PR-0B	ALTAR 84	WX205
13	F_2	*Pina-D1c/Pinb-D1h*	PINAc/PINBh	CIGM88.1209-0B	D67.2/P66.270	TA2462
14	BC_2F_2 and BC_3F_2	*Pina-D1c/Pinb-D1h*	PINAc/PINBh	CIGM90-561	YUK	WX217;TA2462
26	F_2	*Pina-D1c/Pinb-D1h*	PINAc/PINBh	CIGM90.525	ACO89	TA2454
57	F_2	*Pina-D1c/Pinb-D1h*	PINAc/PINBh	CIGM90.812	LCK59.61	TA2460
72	F_2	*Pina-D1c/Pinb-D1h*	PINAc/PINBh	CIGM90.906	SNIPE/YAV79//DACK/TEAL/3	TA2450
74	F_2	*Pina-D1c/Pinb-D1h*	PINAc/PINBh	CIGM90.910	YAV7 2 / TEZ	TA2468
34	F_2	*Pina-D1j/Pinb-D1i*	PINAj/PINBh	CIGM88.1363-0B	DOY1	WX511
29	F_2	*Pina*-D1a/*Pinb*-D1i	PINAa/PINBh	CIGM88.1288-0B	68.111/RGB-U//WARD/3	TA2475

[a] Information from Kansas State Wheat genetics resource center (http://www.k-state.edu/wgrc/)
[b] Pedigree of the durum parent with the subsequent cross being to the *A. tauschii* parent

Table 2. Primers used for *Pina* and *Pinb*.

No. [a]	Gene	Genbank accession	Primer Sequences Forward (5'-3')	Reverse (5'-3')	Size of Product [b]
1	*Pina*	X69913	ATGAAGGCCCTCTTCCTCA	TCACCAGTAATAGCCAATAGTG	446 (25-471)
2	*Pinb*	X69912	ATGAAGACCTTATTCCTCCTA	TCACCAGTAATAGCCACTAGGGAA	446 (17-463)
3	*Pina*	X69913	TAGCGAAGTTGTTGGCAGTT	TTGAGCATCGATCTAGCAGG	109 (87-196)
4	*Pinb*	X69912	GTTTCACAATGAAGGATTTTCCA	TGTGGTGCTATCTGGCTCAG	111 (189-300)
5	*Actin* [c]	AF326781	AAATCTGGCATCACACTTTCTAC	GTCTCAAACATAATCTGGGTCATC	151 (40-190)

[a] 1-2, PCR primers (Gautier et al. 1994); 3-4, qRT-PCR primers; 5 expression control primers for qRT-PCR.
[b] Numbers in the parentheses are positions within the NCBI database accession of primer annealing.
[c] Gene for *Actin* expression (ACT-1) used as an expression control in the qRT-PCR experiment

Selection of Segregating Lines

All synthetic *Ha* locus haplotypes were distinguished from soft type *Ha* locus haplotypes by digesting amplified *Pinb* coding sequence with *Bst*NI. A master mix of enzyme/buffer solution consisting of 0.3 µL 10x *Bst*NI enzyme (New England BioLabs, Ipswich, MA), 0.3 µL enzyme buffer, and 2.4 µL H_2O was added to each 20 µL PCR reaction and the reactions were incubated at 60ºC for 2.5 hrs. Digested PCR products were electrophoresed on 3% MetaPhore high resolution agarose (Lonza, Rockland, ME) gels. The *Pinb*-D1h allele is not digested by *Bst*NI, thus, undigested 447 bp PCR products were scored as *Pinb*-D1h alleles. The *Pinb*-D1a allele is cut once by *Bst*NI resulting in 202 and 245 bp products. Genotypes giving all three possible products after digestion (202, 245, and 447) were scored as heterozygous for the synthetic *Ha* locus. A subset of each genotype were verified by direct sequencing (SeqWright, Houston, TX) of the *Pina* and *Pinb* coding sequences after PCR amplification and gel purification using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Field Testing

The two backcross populations were planted as a randomized complete block with two replications. Each experiment was planted in irrigated and dryland environments in two years at the Montana State University Arthur H. Post Field Research Center near Bozeman, MT on 16 May 2008 and 14 May 2009. The eight populations were grown in randomized block split plot design where main plots were F_2 populations and subplots were individual entries within each cross. The experiment was planted 14 May 2009 at the same site as the backcross populations. All plots were a single 3m row spaced 30 cm

apart seeded with 10 g of seed per plot for the backcross populations and 4 g of seed per plot for the F_2 populations. Prior to harvesting, flowering date was recorded as the days from planting to anthesis. At maturity, seed from all plots was harvested for further analysis.

RNA Expression and qRT-PCR

Total RNA was extracted from 4 of the 'Vanna' background BC_2F_2 derived lines (2 *Pina-D1c/Pinb-D1h* homozygotes and 2 *Pina-D1a/Pinb-D1a Ha* locus homozygotes) from immature seeds harvested 21 days postanthesis (dpa) following the procedure previously described (Hogg et al. 2004). Pure RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI). RT-PCR was performed using a M-MLV reverse transcriptase kit (Promega, Madison, WI). The cDNA template (100 ng) was added to the PCR premix specified by the DyNAmo SYBR Green Two-Step qRT-PCR kit (Finnzymes, Woburn, MA), and expression levels were determined using a MiniOpticon RT-PCR machine (BioRad, Hercules, CA). For the *Pina-D1c/Pinb-D1h* and the *Pina-D1a/Pinb-D1a* lines, loading controls were prepared using RNA isolated from 21 dpa seeds of the soft spring wheat 'Heron' (PI 290910). Specific primers were used for each gene (Table 2) with Actin used as an expression control gene (Wicker et al. 2001). The loading controls for each gene were 0.01x, 0.1x, 1x, and 10x, with 1x equaling 100 ng cDNA template. The fluorescence threshold was set at the level in which the correlation value of the loading series C(t) value and load values in ng was closest to 1. R^2 values ranged from 0.99 for the Actin loading series curve to 0.93 for the *Pina* loading series

curve. The regression line was used to convert sample C(t) values to a relative cDNA load value. *Pina* and *Pinb* levels were compared to the Actin value for each sample.

Total TX-114 and Friabilin Levels

Total TX-114 extractable puroindoline and starch-surface puroindoline (friabilin) levels were quantified from mature seed whole meal prepared from 'Vanna' x Syn14 $BC_2F_{2:3}$ lines that contained either the PINAc/PINBh or the PINAa/PINBa haplotype, 2 F_2 populations ('Vanna' x elite line # 29, with either PINAa/PINBh or PINAa/PINBa, and 'Vanna' x elite line #34, with either PINAj/PINBh or PINAa/PINBa), and parental controls. The PINA null variety, 'McNeal' (PI 574642; Lanning et al. 1994) and the PINB null variety, 'Canadian Red' (CItr 6282; Clark et al. 1926) were also loaded as experimental controls. For TX-114 extractable puroindoline, seeds were ground with a mortar and pestle and puroindolines were extracted from 100 mg of whole seed meal as previously described (Giroux et al. 2003). The extracted proteins were then suspended in 60 µl SDS loading buffer w/o reducing agents (Laemmli, 1970), heated to 70ºC for 10 min and total puroindolines were quantified after electrophoresing on 10 x 10 cm 10-20% Tris-Glycine acrylamide gels (Lonza, Basel, Switzerland) and staining with Coomassie Blue Stain (30% methanol, 10% acetic acid, and 1g/L Brilliant Blue R-250). PINA and PINB were quantified individually after electrophoresing on 14.75% 20 x 20 cm polyacrylamide (acrylamide:Piperazine Diacrylamide ; 30:0.0135; Lamelli, 1970) gels. Gels were silver stained (Bloom et. al., 1987), and PIN bands were quantified visually by comparison with a standard curve created using TX114 proteins extracted from 'Vanna', with 1x equaling 8 µL of extractable protein in SDS loading buffer.

Friabilin was prepared from starch isolated from 200 mg of mature seed whole meal as follows. First, 1 mL 0.1 M NaCl was added to each sample in a 1.5 mL tube and samples were vortexed till suspended before incubating at RT for 30 min. Each sample was then spun for 10 sec at 13,000 g and the flour was worked into a dough ball with a pestle. Next, the starch in suspension was pipetted into a new, pre-weighed 2 mL tube and the dough ball was washed two more times with 0.5 mL 0.1 M NaCl with the supernatant containing suspended starch transferred each time to the same 2 ml tube. The starch suspension was then spun at 13,000 g for 1 min and the supernatant was aspirated. The starch pellet was washed twice by vortexing till suspended in 1 mL 75% w/v $ZnSO_4 \cdot 7H_2O$, then the samples were centrifuged 1 min at 13,000 g, and the supernatant was removed by aspiration (Guraya et. al. 2003). The third high salt solution wash was performed the same as the $ZnSO_4$ washes but used 1 mL 80% w/v CsCl (Guraya et. al. 2003). The starch pellets were then sequentially washed 3 times with 1 mL water and with 1 mL of acetone by vortexing, centrifuging, and aspirating as before. The starch pellet was then allowed to dry and the starch weight was recorded. The starch was resuspended in 400 µL of a 50% isopropanol/0.05 M NaCl solution (Bettge et al. 1995), vortexed at room temperature for 30 min, and centrifuged for 3 min. at 13,000 g. The supernatant was then transferred to a new tube and 1.2 mL acetone was added and the samples were incubated overnight at -20ºC. The samples were then centrifuged for 3 min at 13,000 g, the supernatants were aspirated and the pellets were washed with acetone and allowed to dry. For every 100 mg of starch recovered, 240 µL SDS loading buffer w/o reducing agents (Laemmli, 1970) was added and samples were incubated at 70^{o}C for 10

min. PINA and PINB were quantified relative to a loading control series created using a 'Vanna' friabilin extract where 1x was equal to 12 µL. Proteins were electrophoresed, stained, and quantified using the same 14.75% 20 cm x 20 cm gels described above.

Grain Hardness and Seed Size Traits

Grain hardness and seed size traits were measured using the Single Kernel Characterization System (SKCS) (Perten Instruments, Springfield, IL) with each trait measured on at least 50 clean, unbroken wheat kernels from each field grown replication for the backcross and F2 populations.

Data Analysis

For the backcross populations data for each response variable were analyzed via analysis of variance. The model was that for a randomized complete block design combined over environments. The entries source of variation was partitioned into recurrent parent (Alpowa vs Vanna) *Puroindoline* haplotype (*Pina-D1a/Pinb-D1a* vs *Pina-D1c/Pinb-D1h*), a recurrent parent by *Puroindoline* haplotype interaction and lines within recurrent parent-*Puroindoline* haplotype combination and interactions with environment. The lines within recurrent parent-*Puroindoline* haplotype combination were considered random while other factors were fixed. Analyses were completed using PROC MIXED in SAS. Each F2 population was analyzed separately using a model for randomized complete block using PROC MIXED in SAS. The entries source was partitioned into *Puroindoline* haplotype and lines within *Puroindoline* haplotype class. The lines within *Puroindoline* class was considered a random effect.

Results

Plant and Genetic material

Nine randomly selected accessions of synthetic wheat were obtained from Kansas State University, and *Pina* and *Pinb* were amplified and sequenced using the primers listed in Table 2. Six were found to carry the *Pina-D1c/Pinb-D1h* haplotype while one each possessed the *Pina-D1e/Pinb-D1i*, *Pina*-D1a/*Pinb*-D1i, and the *Pina-D1j/Pinb-D1i* haplotypes (Table 1). At the protein level, the *Pina-D1c/Pinb-D1h* and *Pina-D1e/Pinb-D1i* haplotypes both translate to PINAc/PINBh while *Pina*-D1a/*Pinb*-D1i translates to PINAa/PINBh, and *Pina-D1j/Pinb-D1i* translates to PINAj/PINBh (Figure 1 and Table 1). PINAc contains one amino acid substitution while PINAj shares the same amino acid substitution and contains an additional substitution relative to PINAa. PINBh contains 14 amino acid substitutions relative to PINBa. Elite Synthetic line #14 carries two novel alleles, *Pina-D1c/Pinb-D1h* (PINAc and PINBh), and was chosen to be used in a backcross program with the soft spring wheat varieties 'Alpowa' and 'Vanna' used as recurrent parents with crossing continued to the BC_3F_2 and BC_2F_2 generations, respectively. The remaining eight synthetic accessions were crossed with 'Vanna' and the F_2 generation was studied. *Ha* locus segregation of the BC_2F_2, BC_3F_2, and F_2 populations was checked using a Chi-square test and all ratios were consistent with a single gene 1:2:1 segregation ratio (Table 5).

The $BC_2F_{2:3}$ and $BC_3F_{2:3}$ generations were grown in the field in 2008 and flowering dates were recorded (Appendix). For the $BC_2F_{2:3}$ derived *Pina-D1c/Pinb-D1h* lines in the 'Vanna' background, flowering time did not significantly differ from *Pina-*

D1a/Pinb-D1a. For the $BC_3F_{2:3}$ derived *Pina-D1c/Pinb-D1h* lines in the 'Alpowa' background, flowering time did not differ significantly from *Pina-D1a/Pinb-D1a* lines. Flowering dates were not recorded for the F_2 populations. $BC_2F_{2:4}$ and $BC_3F_{2:4}$ generations were planted in the field in 2009. Agronomic traits were measured including plant height and yield (Appendix). For the $BC_2F_{2:4}$ derived *Pina-D1c/Pinb-D1h* lines in the 'Vanna' background, plant height and yield did not significantly differ from *Pina-D1a/Pinb-D1a*. For the $BC_3F_{2:4}$ derived *Pina-D1c/Pinb-D1h* lines in the 'Alpowa' background, plant height and yield did not differ significantly from *Pina-D1a/Pinb-D1a* lines.

Puroindoline-a

```
PINAa – MKALFLIGLLALVASTAFAQYSEVVGSYDVAGGGGAQQCP
PINAc – ****************************************
PINAj  – ****************************************

PINAa – VETKLNSCRNYLLDRCSTMKDFPVTWRWWKWWKGGCQELL
PINAc – ****************************************
PINAj – ****************************************

PINAa – GECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASK
PINAc – *****Q***********************************
PINAj – *****Q***********************************

PINAa – VIQEAKNLPPRCNQGPPCNIPGTIGYYW
PINAc – ****************************
PINAj – ***************R************
```

Fig. 1. Deduced amino acid sequences of both *Puroindoline*-a and *Puroindoline*-b from nucleic acid sequencing of 'Vanna' as the 'soft type' PINAa/PINBa parent, elite line #34 as the synthetic PINAj/PINBh parent, and elite lines #7, 13, 26, 57, 72, 74 as the synthetic PINAc/PINBh parent. An asterisk (*) indicates conserved identity with the wild type sequence.

Figure 1 continued

Puroindoline-b

```
PINBa- MKTLFLLALLALVASTTFAQYSEVGGWYNEVGGGGGSQQC
PINBh - ********************************A**S****

PINBa - PQERPKLSSCKDYVMERCFTMKDFPVTWPTKWWKGGCEHE
PINBh - *L************GW********F**************

PINBa - VREKCCKQLSQIAPQCRCDSIRRVIQGRLGGFLGIWRGEV
PINBh - ***N******************GM***K****F*****D*

PINBa - FKQLQRAQSLPSKCNMGADCKFPSGYYW
PINBh - **KI************************
```

<u>*Pina* and *Pinb* Transcript Levels</u>

Pina and *Pinb* transcript levels were measured using qRT-PCR on RNA isolated from developing seeds (21 dpa). The mean *Pina* C(t) values for the $BC_2F_{2:3}$ derived lines in the 'Vanna' background were 15.54 for *Pina*-D1c and 14.75 for *Pina*-D1a (Table 3). The relative cDNA amounts for *Pina* were 0.25 for the *Pina-D1c/Pinb-D1h* haplotype and 0.43 for the *Pina-D1a/Pinb-D1a* haplotype when compared to the 'Heron' 'soft type' control. These differences were not significant (Table 3). The mean *Pinb* C(t) values for these same lines were 15.69 for *Pinb*-D1h and 16.80 for *Pinb*-D1a. The relative cDNA amounts for *Pinb* were 0.99 for the *Pina-D1c/Pinb-D1h* haplotype and 0.43 for the *Pina-D1a/Pinb-D1a* haplotype when compared to the 'Heron' 'soft type' control. These differences were also not significant (Table 3).

Table 3. Quantitative real-time PCR C(t) and relative load values of *Pina* and *Pinb* for BC_2F_2 populations.

	C(t) [b]		Relative Load [c]	
Haplotype [a]	*Pina*	*Pinb*	*Pina*	*Pinb*
Vanna (*Pina-D1a/Pinb-D1a*) x Elite #14 (*Pina-D1c/Pinb-D1h*)				
Heron (*Pina-D1a/Pinb-D1a*)	14.61	15.59	1	1
Pina-D1c/Pinb-D1h	15.54 (0.31)	15.69 (0.52)	0.25 (0.15)	0.99 (0.33)
Pina-D1a/Pinb-D1a	14.75 (0.30)	16.80 (0.48)	0.43 (0.14)	0.43 (0.10)
P-value [d]	0.473	0.193	0.499	0.247

[a] Vanna by *Pina-D1c/Pinb-D1h*. N for both *Pina-D1c/Pinb-D1h* and *Pina-D1a/Pinb-D1a* was 2. (Means are given with standard error in parentheses). Heron was used as a 'soft type' control.

[b] C(t) value was determined by the MiniOpticon (BioRad, Hercules, CA) as the point when inflorescence reached a preset threshold.

[c] Relative load value is the relative amount of cDNA compared to a loading curve of 0.01x, 0.1x, 1x, and 10x of 'Heron' 'soft type' control. The number represents the Actin 1x value divided by the Actin sample value multiplied by Pin sample value. 'Heron' values were given a value of one with the 'soft type' and synthetic haplotypes compared to them.

[d] *P*-value was calculated using a studentized t-test comparing 'soft type' haplotype means to synthetic haplotype means.

PINA and PINB Protein Expression Levels

To assess the effect of the different *Ha* haplotypes upon PINA and PINB expression and degree of starch binding, PINs were extracted from mature seeds with TX-114 to estimate total extractable PIN and from the surface of starch to assess the degree of PIN functionality. Total extractable (TX-114) and starch bound levels of PINA and PINB were then visually assessed using SDS-PAGE (Fig. 2 and 3, respectively). The null control varieties, 'McNeal' (PINA null, PINBa) and 'Canadian Red' (PINAa, PINB null), were used to show the separation of the PINA and PINB bands. In the 'Vanna' parental controls, PINAa is more abundant with its level roughly twice that of PINBa in terms of both total expression (Fig 2) or as levels bound to starch (Fig 3). The PINAa/PINBh, PINAc/PINBh, and PINAj/PINBh proteins extracted from individuals homozygous for the synthetic haplotype show a more abundant band in the location of the 'soft type' PINB band with a less abundant band running slightly faster (Fig 2 and 3). N-terminal sequencing determined that these two bands were in fact PINA and PINB respectively (results not shown), leading to the conclusion that the novel *Ha* PINs have altered mobility but similar expression levels to the PINAa/PINBa proteins of the 'Vanna' parental controls or the PINAa/PINBa locus segregants (Fig. 2). The intensity of these bands was quantified visually and given a value based on the relative intensity compared to the loading control sequence (Table 4). The 'Vanna' loading control bands for total endosperm puroindoline, PINA, and PINB were each set at 1.0 and the experimental samples were given values in relation to the 'Vanna' controls.

There were no significant differences in either total TX-114 extractable or starch bound levels of PINA and/or PINB between synthetic allele groups and soft type allele groups. Levels of PINA in the $BC_2F_{2:3}$ derived lines with the 'Vanna' recurrent parent varied up to 20% relative to the wild type controls while in the F_2 derived lines with the PINAa/PINBh haplotype, the mean PINA and PINB values were also within 20% of the wild type values. In the $F_{2:3}$ derived lines with the PINAj/PINBh haplotype the mean TX-114 extractable and starch bound PINA and PINB levels were within 30% of the control values. While this variation may be enough to cause differences in grain texture, the differences were not significant. Both PIN bands present for the synthetic *Ha* loci appeared lower on the gel than the PIN bands present for the *Pina-D1a/Pinb-D1a Ha* locus.

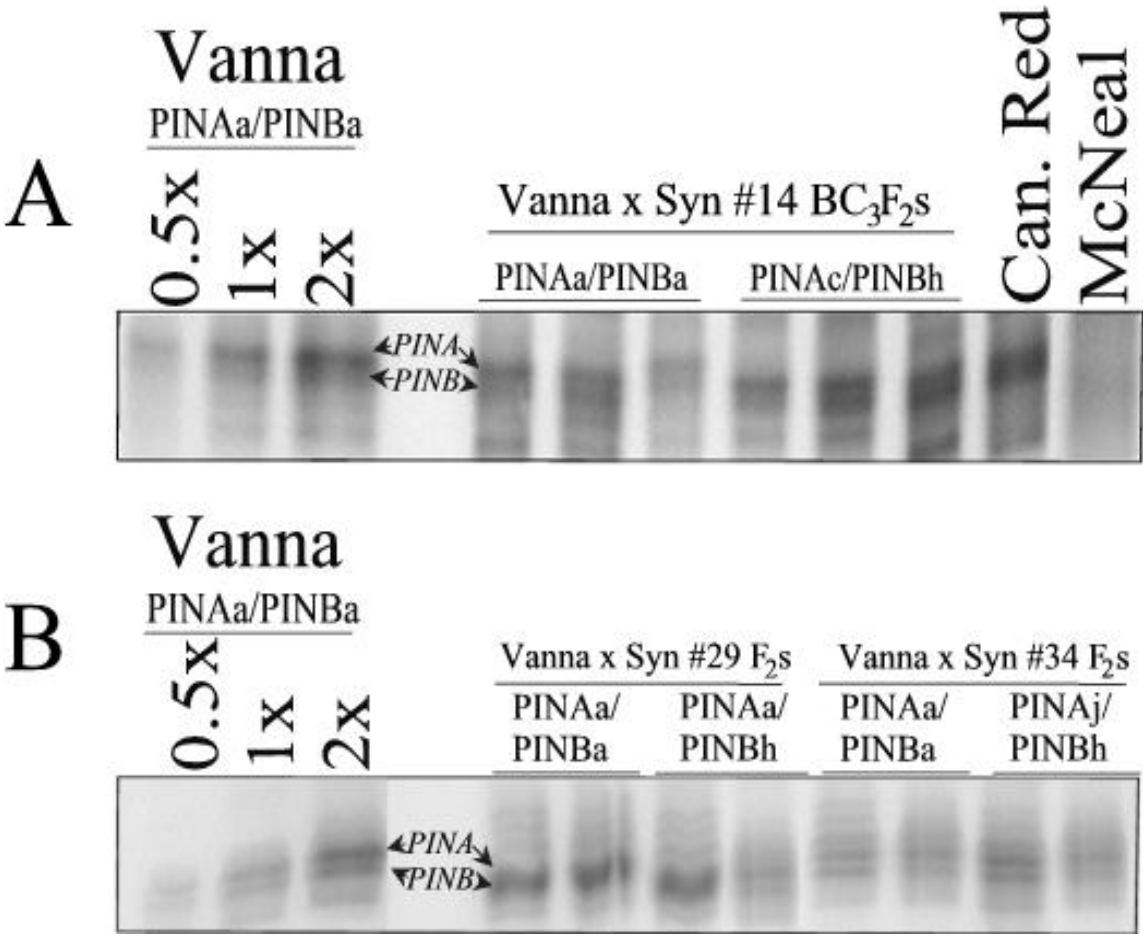

Fig. 2. A) TX-114 extracted endosperm puroindolines for 'Vanna' by PINAc/PINBh cross. Canadian Red is a PINB null control and McNeal is a PINA null control. There are three representative independent $BC_2F_{2:3}$ lines shown for the PINAa/PINBa samples and for the PINAc/PINBh samples in this population. B) TX-114 extracted endosperm puroindolines for 'Vanna' by PINAa/PINBh and PINAj/PINBh crosses. There are two representative independent $F_{2:3}$ lines for the PINAa/PINBa samples and for the PINAa/PINBh samples in the Vanna x Syn #29 population, and there are two representative independent $F_{2:3}$ lines for the PINAa/PINBa samples and for the PINAj/PINBh samples in the Vanna x Syn #34 population.

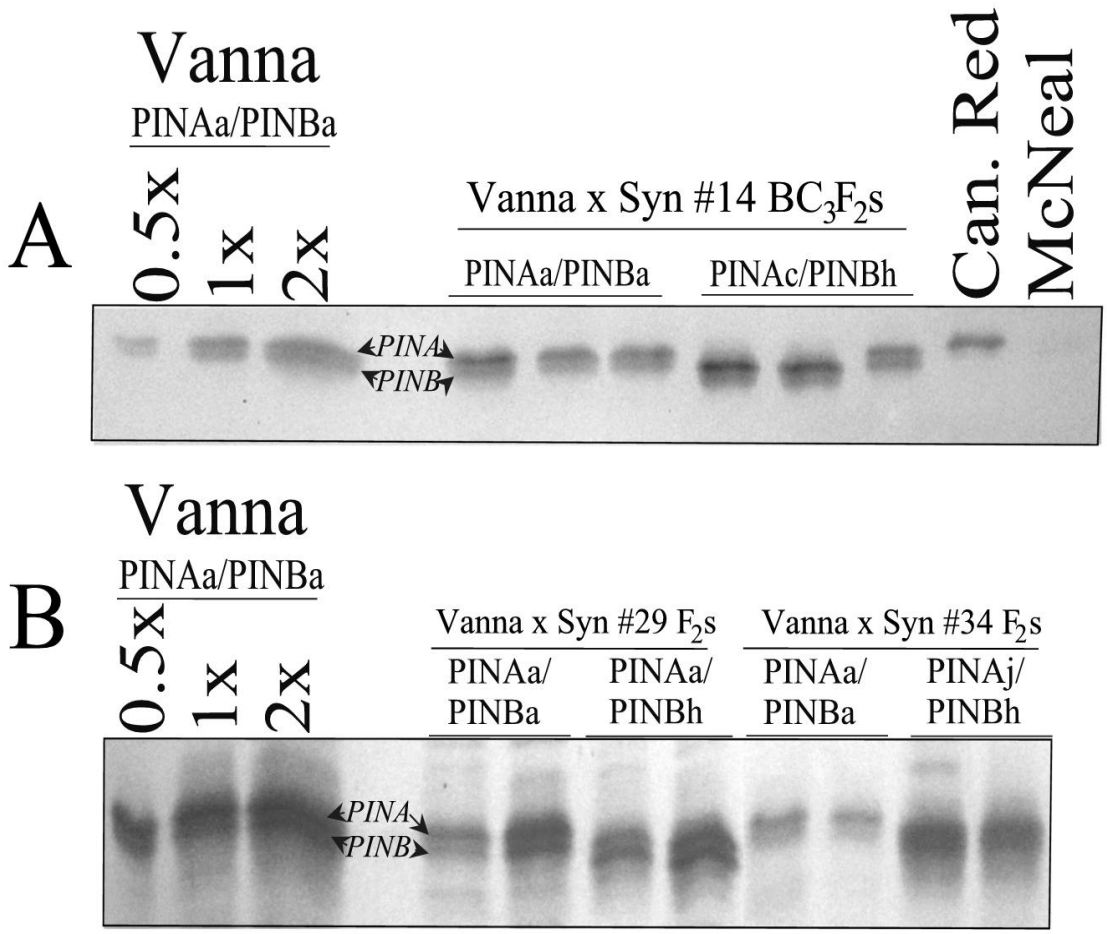

Fig. 3. A) Friablin extracted from starch showing separated puroindolines for 'Vanna' by PINAc/PINBh cross. Canadian Red is a PINB null control and McNeal is a PINA null control. The same samples were used here as for the total endosperm puroindolines in figure 2. B) Friabilin extracted from starch showing separated puroindolines for 'Vanna' by PINAa/PINBh and PINAj/PINBh crosses. The same samples were used here as for the total endosperm puroindolines in figure 2.

Table 4. *Puroindoline* expression levels, total puroindoline content, and bound puroindoline content (friabilin) for positive and negative synthetic lines.

	Total PINA [b,e]	Total PINB [b,e]	Total PIN [b,e]	Friabilin PINA [c,e]	Friabilin PINB [c,e]	Total Friabilin [c,e]
'Vanna' [a]	1	1	1	1	1	1
Haplotype [d]						
$BC_2F_{2:3}$ 'Vanna' (*Pina-D1a/Pinb-D1a*) x Elite #14 (*Pina-D1c/Pinb-D1h*)						
PINAc/PINBh	1.2(0.21)	1.3(0.26)	1.3(0.22)	1.0(0.24)	1.0(0.27)	1.0(0.26)
PINAa/PINBa	0.9(0.18)	1.1(0.22)	1.0(0.22)	1.2(0.39)	1.2(0.15)	1.2(0.24)
P-value	0.073	0.383	0.242	0.635	0.243	0.361
$F_{2:3}$ 'Vanna' (*Pina-D1a/Pinb-D1a*) x Elite #29 (*Pina*-D1a/*Pinb*-D1i)						
PINAa/PINBh	1.2(0.14)	1.1(0.14)	1.2(0.09)	1.0(0.07)	1.0(0.14)	1.0(0.10)
PINAa/PINBa	1.1(0.00)	1.1(0.07)	1.1(0.04)	0.9(0.14)	1.0(0.28)	1.0(0.19)
P-value	0.423	0.698	0.617	0.698	0.998	0.629
$F_{2:3}$ 'Vanna' (*Pina-D1a/Pinb-D1a*) x Elite #34 (*Pina-D1j/Pinb-D1i*)						
PINAj/PINBh	1.3(0.21)	1.2(0.07)	1.2(0.14)	1.00(0.00)	1.0(0.35)	0.97(0.05)
PINAa/PINBa	1.25(0.14)	1.3(0.07)	1.3(0.07)	0.95(0.21)	1.1(0.07)	1.0(0.24)
P-value	0.808	0.293	0.956	0.771	0.733	0.701

[a] The bands in the 'Vanna' 1x loading control were given a value of 1 and all other lines were given a value relative to this.
[b] TX-114 extracted *Puroindoline* from grain endosperm was quantified using a 0.5x to 2x scale. Number is the average of two experiments.
[c] Starch bound PINA and PINB was quantified using a 0.5x to 2x scale.
[d] Results from protein expression of 'Vanna' by *Pina-D1c/Pinb-D1h* ($BC_2F_{2:3}$) , *Pina-D1a/Pinb-D1i* ($F_{2:3}$) , and *Pina*-D1j/*Pinb*-D1h ($F_{2:3}$) crosses.
[e] 1.0 was the value assigned to the 1x band for the total, PINA and PINB bands of the 'Vanna' parent since they are not being compared to each other in this study. Standard errors are in parentheses. *P*-value obtained using a studentized t-test.

Grain Texture

Overall, $BC_3F_{2:3}$ seeds homozygous for the *Pina-D1c/Pinb-D1h* haplotype in the 'Alpowa' background had a mean hardness of 36.6 in 2008 and 33.8 in 2009 and were significantly harder, by 9.0 and 9.5 SKCS hardness units, respectively, than $BC_3F_{2:3}$ seeds homozygous for the *Pina-D1a/Pinb-D1a* haplotype (P=0.020 and P<0.001). *Pina-D1c/Pinb-D1h* homozygous seeds in the 'Vanna' background had a mean hardness of 37.5 in 2008 and 28.1 in 2009 and were also significantly harder (P=0.052 and P<0.001, repectively) than the *Pina-D1a/Pinb-D1a* homozygous seeds, which has a mean of 30.2 in 2008 and 16.2 in 2009. In both years, *Pina-D1c/Pinb-D1h* homozygous seeds showed no significant difference in kernel weight compared to the 'soft type' haplotypes in either background (Table 5).

SKCS hardness was then analyzed on the remaining eight haplotypes in the F_2 generation in order to determine if any significant differences in function could be attributed to the different *A. tauschii* donors, the different durum donors, or to the different synthetic haplotypes (Table 6). In all five F_2 populations segregating for the *Pina-D1c/Pinb-D1h* synthetic haplotype, the synthetic *Ha* haplotype was associated with harder texture than seeds with the *Pina-D1a/Pinb-D1a* haplotype. The differences ranged from an average of 3.8 units for the 'Vanna' x Elite line #52 to 7.7 units for the 'Vanna' x elite line #26 population (Table 7). While not all five of the differences were significant at the 0.05 level, when the five populations were combined, the mean hardness for the *Pina-D1c/Pinb-D1h* haplotype was 31.0 compared to the mean for the *Pina-D1a/Pinb-D1a* haplotype (25.8), was significant (P<0.001). The population segregating for the

Pina-D1e/Pinn-D1i haplotype also had a significant difference in SKCS hardness. The *Pina*-D1e/Pinn-D1i conferred a 5.6 unit increase over the *Pina-D1a/Pinb-D1a* haplotype (*P*=0.028). The two populations segregating for additional *Ha* locus haplotypes gave similar results in that in each case the synthetic *Ha* locus was associated with increased grain hardness. In the Vanna x elite synthetic line #34, the *Pina*-D1j/*Pinb*-D1h haplotype group was 3.8 units harder than the seeds with the *Pina-D1a/Pinb-D1a* haplotype. While this difference was not significant it did show the same trend as the rest of the populations. In the Vanna x elite synthetic #29 population, the *Pina-D1a/Pinb-D1i* group was 12.6 units harder (*P*<0.001) than the group having the *Pina-D1a/Pinb-D1a* haplotype. No significant differences in kernel weight were seen between the synthetic and 'soft type' *Ha* locus groups in any population (Table 5 and 6). SKCS was also run in 2008 and showed similar results (data not shown).

Table 5. Grain hardness and kernel weight for BC_3F_2 and BC_2F_2 derived populations from elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with 'Alpowa' and 'Vanna' (*Pina*-D1a/*Pinb*-D1b) soft white spring wheats.

Ha locus genotype	Segregation Data	2008		2009	
		SKCS Grain Hardness [a]	Kernel weight (mg) [a]	SKCS Grain Hardness [a]	Kernel weight (mg) [a]
	BC_3F_2	Alpowa*4/Elite line #14			
Pina-D1c / *Pinb*-D1h	4	36.6 (1.1)	41.0 (0.8)	33.8 (1.0)	35.9 (0.9)
Heterozygote	10	-	-	-	-
Pina-D1a / *Pinb*-D1a	3	27.6 (1.4)	40.2 (0.7)	24.4 (1.5)	36.4 (0.7)
P-value [b,c]	0.724	0.020	0.446	<0.001	0.642
	BC_2F_2	Vanna*3/Elite line #14			
Pina-D1c / *Pinb*-D1h	3	37.5 (1.8)	35.0 (0.7)	28.1 (1.2)	36.4 (1.0)
Heterozygote	15	-	-	-	-
Pina-D1a / *Pinb*-D1a	4	30.2 (2.5)	36.2 (0.9)	16.2 (1.1)	36.4 (0.9)
P-value	0.223	0.052	0.326	<0.001	0.999

[a] Mean represents average of two replications in two environments with standard error in ().
[b] *P*-value for the segregation data is based on a Chi-squared test for single gene segregation model
[c] *P*-value for hardness and kernel weight is based on studentized t-test.

Table 6. Single Kernel Characterization System (SKCS) and segregation data of F_2 populations.

Ha locus genotype	Segregation Data [a]	SKCS Grain Hardness [a]	Kernel weight (mg) [a]
	'Vanna' x Elite #13 *Pina-D1c/Pinb-D1h*		
Pina-D1c / *Pinb*-D1h	15	28.2 (1.6)	38.3 (0.9)
Heterozygote	30	-	-
Pina-D1a / *Pinb*-D1a	26	23.9 (1.3)	38.6 (0.9)
P-value [b,c]	0.078	0.042	0.755
	'Vanna' x Elite #26 *Pina-D1c/Pinb-D1h*		
Pina-D1c / *Pinb*-D1h	11	37.4 (2.1)	35.8 (0.9)
Heterozygote	22	-	-
Pina-D1a / *Pinb*-D1a	14	29.7 (3.1)	35.8 (0.5)
P-value	0.75	0.048	0.142
	'Vanna' x Elite #57 *Pina-D1c/Pinb-D1h*		
Pina-D1c / *Pinb*-D1h	21	25.1 (1.3)	40.7 (0.8)
Heterozygote	41	-	-
Pina-D1a / *Pinb*-D1a	17	21.3 (1.5)	40.1 (0.9)
P-value	0.771	0.063	0.61
	'Vanna' x Elite #72 *Pina-D1c/Pinb-D1h*		
Pina-D1c / *Pinb*-D1h	15	30.8 (2.3)	39.8 (0.7)
Heterozygote	35	-	-
Pina-D1a / *Pinb*-D1a	22	25.4 (2.8)	39.2 (0.9)
P-value	0.492	0.145	0.537
	'Vanna' x Elite #74 *Pina-D1c/Pinb-D1h*		
Pina-D1c / *Pinb*-D1h	13	33.6 (1.5)	39.6 (0.9)
Heterozygote	36	-	-
Pina-D1a / *Pinb*-D1a	14	28.7 (1.4)	39.6 (1.1)
P-value	0.518	0.030	0.989

(Table 6 continued)

'Vanna' x *Pina-D1c/Pinb-D1h* Combined			
Pina-D1c / *Pinb*-D1h	75	31.0 (0.8)	38.5 (0.4)
Heterozygote	164	-	-
Pina-D1a / *Pinb*-D1a	93	25.8 (0.9)	38.9 (0.5)
P-value	0.368	<0.001	0.570
'Vanna' x Elite #7 *Pina-D1e/Pinb-D1i*			
Pina-D1e / *Pinb*-D1i	21	32.8 (1.7)	32.6 (0.6)
Heterozygote	37	-	-
Pina-D1a / *Pinb*-D1a	30	27.2 (1.2)	34.1 (0.8)
P-value	0.131	0.028	0.169
'Vanna' x Elite #29 *Pina*-D1a/*Pinb*-D1i			
Pina-D1a / *Pinb*-D1i	25	39.0 (1.4)	35.2 (0.5)
Heterozygote	39	-	-
Pina-D1a / *Pinb*-D1a	18	26.4 (1.7)	36.3 (0.6)
P-value	0.499 [b]	<0.001[¶]	0.153[¶]
'Vanna' x Elite #34 *Pina-D1j/Pinb-D1i*			
Pina-D1j / *Pinb*-D1i	17	28.4 (1.6)	38.6 (0.8)
Heterozygote	35	-	-
Pina-D1a / *Pinb*-D1a	17	24.6 (1.6)	37.8 (1.1)
P-value	0.993 [b]	0.103	0.568[¶]

[a] Mean represents average of four replications in 2009 with standard error in ().
[b] *P*-value for the segregation data is based on a Chi-squared test
[c] *P*-value for hardness and kernel weight is based on studentized t-test and analyzed using the PROC MIXED procedure in SAS.

Table 7. Hardness differences based on haplotype.

Ha locus haplotype [a]	SKCS hardness increase [c]
Pina-D1c / *Pinb*-D1h [b]	5.2a
Pina-D1e / *Pinb*-D1i	5.6a
Pina-D1j / *Pinb*-D1i	3.8a
Pina-D1a / *Pinb*-D1i	12.6b

[a] Synthetic haplotype that confers increased hardness compared to the 'soft type' haplotype in the same F_2 population.
[b] *Pina-D1c/Pinb-D1h* difference represents the mean differences of all five populations with this synthetic haplotype.
[c] Increase compared to the samples with the *Pina-D1a/Pinb-D1a* haplotype. Mean differences followed by the same letter do not differ significantly.

Discussion

The objective of this study was to test the impact of the *Pina-D1c/Pinb-D1h* haplotype in near isogenic lines, and to examine the *Pina-D1c/Pinb-D1h* haplotype and other unique *Ha* haplotuypes in segregating populations. Gedye et al. (2004) reported the *Pina-D1c/Pinb-D1h* haplotype was associated with decreased grain hardness. In Gedye's study, the PINA and PINB alleles were described independent of each other and all hardness testing was done on the original synthetic parents, leading to possible background effects (Gedye et al. 2004). Here, *Pina-D1c/Pinb-D1h* was introduced into common wheat cultivars, which was specifically done by analyzing the haplotypes found in 9 randomly chosen synthetic hexaploid accessions (Massa et al. 2004), and crossing them into the soft white spring wheat varieties 'Alpowa' and 'Vanna'. Sequence analysis of these accessions revealed 4 novel *Ha* locus haplotypes (Table 1 and Fig. 1). Seven had the synthetic protein haplotype PINAc/PINBh, the haplotype previously associated with increased grain softness in a survey of synthetic wheats (Gedye et al. 2004). By examining this haplotype in crosses with soft common hexaploid wheat cultivars and in more molecular detail, the hope was to try to ascertain the true functional effect of this haplotype. The other two synthetic accessions had the protein haplotypes PINAa/PINBh and PINAj/PINBh (Table 1 and Figure 1). These haplotypes were also analyzed for their effects on grain texture. One PINAc/PINBh accession had its *Ha* locus backcrossed into two different soft wheat varieties in order to reduce the amount of non-*Ha* locus linked genes from the durum or *A. tauschii* donor. The remaining eight accessions were crossed

with the soft white wheat variety, 'Vanna', and the populations were analyzed as F_2's. This was done to try and measure the amount of background effect attributable to the durum or *A. tauschii* donors, as well as to test the novel haplotypes, PINAc/PINBh and PINAj/PINBh.

The effect of the unique *Ha* locus haplotypes upon *Pina* and *Pinb* transcript levels was assessed with qRT-PCR (Table 3). Previous studies have used northern blot analysis to quantify RNA expression (Giroux and Morris, 1997). Real-time PCR allows for higher throughput (Heid et al. 1996). Heid et al. (1996) also showed that equal amounts of input DNA can give up to a 1 cycle standard deviation, which equates to almost a 2 fold difference in expression. Therefore, greater than 2-fold increase or decrease in expression is required for detection. Our results indicated that there was little to no difference in *Pina* or *Pinb* transcript levels between synthetic and 'soft type' lines in the $BC_2F_{2:3}$ *Pina-D1c/Pinb-D1h* population (Table 3).

With no apparent differences in RNA expression, a test for the abundance and function of PINA and PINB, was a logical subsequent step, and was done on all three of the *Ha* haplotypes. By extracting puroindolines from the endosperm of a seed using TX-114, the absolute level of PINA and PINB in seeds can be assessed (Blochet et al. 1991, 1993; Giroux et al. 2003), while the level of PINA and PINB bound to the surface of starch gives a measure of their function (Bettge et al. 1995). In this way, the effect of each unique *Ha* haplotype upon PIN expression level and function was assessed (Fig. 2 and 3, Table 4). None of the synthetic haplotypes was associated with changes in PIN expression or starch binding. If small differences were observed in starch binding, it

would be predicted that hardness would increase since reduced abundance of either PIN bound to starch increases grain hardness since both PINs interacting with each other is necessary to create friabilin (Hogg et al. 2004; Wanjugi et al. 2007; Martin et al. 2007; Zhang et al. 2009). In this study, it was determined that total friabilin was not significantly reduced in populations that had the synthetic haplotype (Table 4).

While PIN expression levels were unchanged, each synthetic *Ha* haplotype resulted in altered mobility of both PINA and PINB (Fig. 2 and 3). This leads to the conclusion that the altered mobility results from a conformational change in the synthetic proteins relative to PINAa and PINBa, which is plausible given that the protein samples were run on polyacrylamide gels in the absence of reducing agents. Because puroindolines account for 60% of the hardness differences seen in crosses between hard and soft wheats (Campbell et al. 1999), it would be plausible that even though no expression differences were seen, some functional difference may be observed.

This is the first study to report functional differences between different soft *Ha* loci. The 'soft type' wheat cultivars 'Alpowa' and 'Vanna' were used as recurrent parents in crossing with synthetic wheats carrying the *Pina-D1c/Pinb-D1h* haplotype. Because the populations were not isogenic, the effects of linked genes upon grain hardness cannot be excluded. Our results differ from those of Gedye et. al. (2004) who reported that the *Pina-D1c/Pinb-D1h* alleles were each associated with softer grain texture, likely due to other background effects from the durum or *A. tauschii* donor. Our results differ in finding that the PINAc/PINBh, PINAj/PINBh, and PINAa/PINBh haplotypes confer a 4 to 13 unit increase in hardness (Table 5 and 6). Severe mutations in PINA or PINB

increase SKCS hardness by 30-60 units (Giroux and Morris, 1997, 1998). Soft wheats typically fall between 24 and 37 hardness units and hard wheats typically fall between 63 and 86 hardness units (Pearson et al. 2009), indicating that the synthetic haplotypes studied here still confer soft texture. This also indicates some remaining function within PINA and PINB in all tested *Ha* locus haplotypes. Interestingly, the difference seen in the F_2 population with the *Pina*-D1a/*Pinb*-D1i *Ha* haplotype shows a much greater increase in hardness compared to the other populations (Table 7). This leads to the possibility that the PINBh protein allele is directly responsible for these increases and the PINAc and PINAj protein alleles may be decreasing hardness. This wasn't possible to test because *Pinb*-D1a is never paired with a novel *Pina* allele in any of the available synthetic wheats (Gedye et al. 2004, Table 1). Presence of friabilin proteins (Fig. 3) helped to support this idea, as friabilin is scarce in starch isolated from hard wheat varieties (Greenwell and Schofield, 1986). The relatively small functional differences observed via comparisons of SKCS grain hardness values indicates that only small differences in expression or function of the proteins exist, making them difficult to observe. This is consistent with the fact that we were not able to detect changes in expression at the RNA (Table 3), protein (Table 4), or starch binding level (Table 4).

Because for the PINAa/PINBh haplotype, the PINA allele is wild type, the hardness change in that cross or in others can't be attributed to PINA but rather to PINBh. The PINBh allele contains 14 missense mutations relative to the PINBa allele. Slight decreases in PINB function are more likely to be observed since PINB limits grain softness more than PINA (Swan et. al. 2006). Overexpression of both PINA and PINB

has shown increases in lipid content (Feiz et al. 2009b). PINBh may limit grain softness via decreases in lipid binding since puroindolines interaction with starch is mediated by lipids (Greenblatt et al. 1995). Additional experimentation is required to investigate the effect of different synthetic wheat derived *Ha* haplotypes upon starch lipid levels.

Conclusion

Because each of the synthetic wheat derived *Ha* locus haplotypes increased grain hardness, they could prove useful in modifying soft wheat milling and baking quality. Testing the BC_2F_2 population for milling and baking qualities, such as flour yield, starch damage, ash content, solvent retention and cookie diameter would give some insight into end use possibilities of these synthetic haplotypes. The creation of near-isogenic lines in several soft wheat backgrounds could also give more data as to the true effect of these novel haplotypes. This study has found several synthetic wheat derived *Ha* locus haplotypes which increase grain hardness of soft wheat. In particular, the *Pinb*-D1h allele is associated with increased grain hardness. Further experiments into the effects of these novel synthetic *Ha* loci on milling and baking quality could provide valuable information for practical use.

CHAPTER 2

NOVEL HA LOCUS, *PINA-D1C/PINB-D1H*, IMPACTS SOFT WHITE SPRING WHEAT MILLING AND BAKING CHARACTERISTICS

Introduction

Classification of hexaploid wheat (*Triticum aestivum* L.) varieties depends a great deal on grain hardness (reviewed in Morris and Rose, 1996). The *Ha* locus, which contains three linked genes, *Puroindoline*-a (*Pina*) (Sourdille et al. 1996; Giroux and Morris, 1998), *Puroindoline*-b (*Pinb*) (Giroux and Morris, 1997), and Grain softness protein (Gsp-1) (Jolly et al. 1996), is responsible for 60% of hardness differences between soft and hard wheats (Campbell et al. 1999). Surveys of *Pina* and *Pinb* alleles present in hexaploid wheat varieties have found that all soft wheats contain the *Ha* locus haplotype *Pina-D1a/Pinb-D1a*, while all hard wheats contain a point mutation in either *Pina* or *Pinb* (Giroux and Morris, 1998). Hard wheats that vary in Pin alleles vary in grain hardness and end product quality (Martin et al. 2001, 2008). Therefore, in order to improve soft wheat quality it is important to have a great deal of *Ha* locus diversity. A survey of the diploid wheat relative, Aegilops tauschii, found nine new *Pina*-D1/*Pinb*-D1 haplotypes (Massa et al. 2004). These haplotypes can also be found in synthetic accessions of hexaploid wheat, created via the hybridization between the tetraploid Triticum turgidum (AABB) and *A. tauschii* (DD; Mujeeb-Kazi et al. 1996). A synthetic wheat survey of four of these new haplotypes (*Pina-D1c/Pinb-D1h*, *Pina-D1e/Pinb-D1i*, *Pina*-D1a/*Pinb*-D1i, and *Pina-D1j/Pinb-D1i*) demonstrated that all four are functional *Ha* loci that confer soft phenotypes (Gedye et al. 2004). In crosses with hexaploid wheats

each of the new *Ha* haplotypes increased grain hardness with differences ranging from 4-12 Single Kernel Characterization System (SKCS) units versus the *Pina-D1a/Pinb-D1a* haplotype (Chapter 1).

Soft wheat is generally used in making cakes and cookies while hard wheat is generally used for making bread (Rogers et al. 1993). Bread quality is often related to the amount and quality of protein that is present, and specifically to the high molecular weight glutenins (Payne et al. 1984). It has also been shown that phospholipids and glycolipids can increase bread loaf volume (MacRitchie and Gras, 1973), and it is believed that the PIN proteins are involved in the binding of these lipids to the surface of starch granules (Greenblatt et al. 1995; Feiz et al. 2009b). Besides lipid binding, the major grain hardness quality associated traits for soft wheats include starch damage, particle size and size distribution after milling, which affect swelling, pasting, and gelling qualities (Dengate et al. 1984). Ultimately, increasing softness among soft wheats is associated with increased cookie diameter and decreased sucrose retention capacity (Gaines, 2000; Guttieri et al. 2001). The novel *Ha* loci from synthetic wheat accessions are some of the only naturally occurring functional *Ha* loci and represent an opportunity to better understand the effects of minor hardness variation within soft wheats upon soft wheat milling and baking quality.

Here, synthetic wheat haplotype *Pina-D1c/Pinb-D1h* was backcrossed into two soft white spring wheat varieties. The two populations were then analyzed for their milling qualities by measuring their protein content and flour particle size distribution after milling. End product quality of flour was then analyzed by solvent retention

capacities (SRC, AACC method 56-11; Gaines, 2000; Slade and Levine, 1994), and alkaline water retention capacity (AWRC, AACC method 56-10). Then baking quality was tested by analyzing sugar snap cookie diameters, heights, and appearance.

Materials and Methods

Plant Material

Seeds of elite synthetic line #14, which has the *Ha* haplotype *Pina-D1c/Pinb-D1h*, were obtained from the Kansas State Wheat Genetic Resource Center and planted in the greenhouse along with the soft white spring wheat varieties 'Alpowa' (PI 566596) (Released in 1994 by Washington State University) and 'Vanna' (PI 587199) (Released in 1995 by Western Plant Breeders Inc), both of which have the *Ha* haplotype *Pina-D1a/Pinb-D1a*. Initial crosses were made with 'Alpowa' and 'Vanna' as the female parents and the synthetic line as the male parent.

Selection of Segregating Lines

F_1's were planted and DNA was extracted from leaf tissue of individual plants (Riede and Anderson, 1996). DNA was screened by amplifying only *Pinb* (Primers in Table 1) digesting the product with *Bst*NI, and running the fragments on high resolution agarose gels (Reynolds et al. 2009) in order to identify *Pinb*-D1a/*Pinb*-D1h heterozygotes for crossing. Subsequently, the 'Alpowa' population was backcrossed 3 more times, and the 'Vanna' population was backcrossed 2 more times, each with the soft white spring wheat as the recurrent parent. After each cross, F_1's were screened to identify *Ha* locus heterozygotes. Both populations were then allowed to self and BC_3F_2's and BC_2F_2's were

screened for individuals homozygous for either the *Pina-D1a/Pinb-D1a* or the *Pina-D1c/Pinb-D1h* locus (Reynolds et al. 2009). All BC_3F_2's and BC_2F_2's *Ha* loci were also verified by direct sequencing (SeqWright, Houston, TX) of the *Pina* and *Pinb* coding sequences (Primers in Table 1) after PCR amplification and gel purification using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Field Testing

The two backcross populations were evaluated in separate adjacent field trials. Each experiment was a randomized complete block with two replications. All plots were a single 3m row spaced 30 cm apart seeded with 10 g of seed. Each experiment was planted in irrigated and dryland environments at the Montana State University Arthur H. Post Field Research Center near Bozeman, MT on 14 May 2009. The irrigated environment received an additional 6.25 cm of water two weeks prior to anthesis. At maturity, seed from all plots was harvested for milling and baking analyses.

End Quality Testing

Three lines with the *Pina-D1a/Pinb-D1a* and four with the *Pina-D1c/Pinb-D1h Ha* locus haplotypes from the 'Alpowa' population from all four replications were tested for hardness using the Single Kernel Characterization System (SKCS, Perten Instruments, Springfield, IL). The same was done for 4 lines with *Pina-D1a/Pinb-D1a* and 3 lines with *Pina-D1c/Pinb-D1h Ha* locus haplotypes from the 'Vanna' population from all four replications. One hundred grams of seed were then tempered to 14% moisture 18 to 24 hours before being milled on a Quadramat Jr. mill (C.W. Brabender Instrument, Inc., South Hackensack, NJ). Bran and flour were weighed individually and flour yield was

calculated as the proportion of break flour and reduction flour to total products. Then, flour was separated using 150, 75, and 53 µm USA standard sifting screens and the sieve products were weighed. Near-infrared reflectance (NIR, InfraTec GmbH, Dresden, Germany) was run on the flour to test protein (which is converted from nitrogen content by multiplying by 6.25), moisture, and ash content.

Flour solvent retention capacities (SRCs, AACC Method 56-11, Slade and Levine, 1994; alkaline water retention capacity, AACC Method 56-10) were also measured. The five solvents were deionized water, 50% (w/w) sucrose solution, 5% (w/w) sodium carbonate solution, 5% (w/w) lactic acid solution, and an alkaline water solution (0.1 N sodium bicarbonate). The following modifications were made to the approved AACC method. After pre-weighing 2 mL microcentrifuge tubes, 200 mg of flour was weighed out and added to each tube along with 1 mL of solvent. The tubes were vortexed for 2 min in order to disperse the flour, and then the samples were rested for 18 minutes with 5 seconds of vigorous shaking at 5, 10, 15, and 20 minutes after the addition of the solvent. After 20 minutes, the tubes were centrifuged for 15 min at 1000 x g. The supernatants were decanted and the tubes were drained at a 90^{o} angle for 10 min. SRC values were calculated as described in AACC method 56-11.

Baking quality was tested using the AACC sugar snap cookie baking procedure (AACC Method 10-52). Quality was determined through the measurement of four characteristics; cookie spread (average diameter of two cookies); cookie height (stack height of two cookies); weight loss upon baking, and top grain score, or the extent of islanding on the cookie surface compared to a standard set of cookies ranging from a

score of 0 to 9. Cookies given a score of 0 have smooth surface indicating poor baking quality. Islanding, and with it baking quality, increases as the top grain score increases.

Data Analysis

Data for each response variable were analyzed via analysis of variance. The model was that for a randomized complete block design combined over environments. The entries source of variation was partitioned into recurrent parent ('Alpowa' vs 'Vanna'), *Puroindoline* haplotype (*Pina-D1a/Pinb-D1a* vs *Pina-D1c/Pinb-D1h*), a recurrent parent by *Puroindoline* allelic class interaction, lines within recurrent parent-*Puroindoline* haplotype combination, and interactions with environment. The lines within recurrent parent-*Puroindoline* allelic class combination were considered random while other factors were fixed. Analyses were completed using PROC MIXED in SAS.

Results

The soft white spring wheat varieties 'Alpowa' and 'Vanna' having the *Pina-D1a/Pinb-D1a Ha* haplotype were used as recurrent parents in crosses with Elite Synthetic #14 having the *Pina-D1c/Pinb-D1h* haplotype. Individual BC_3F_2 ('Alpowa' parent) and BC_2F_2 ('Vanna' parent) lines were genotyped by amplifying *Pinb* and digesting with *Bst*NI to identify individual plants homozygous for either of the two segregating *Ha* haplotypes. The *Ha* haplotypes of all lines were confirmed by sequencing of *Pina* and *Pinb* coding sequences. *Ha* locus segregation in both the 'Alpowa' (total of 17 individuals tested) and 'Vanna' (total of 22 individuals tested) backgrounds was not significantly different from the expected 1:2:1 ratio for normal single gene segregation as

determined by a Chi-square test. In the ‘Alpowa’ background population, there were 4 *Pina-D1a/Pinb-D1a* and 3 *Pina-D1c/Pinb-D1h Ha* locus homozygotes. In the ‘Vanna’ population there were 3 *Pina-D1c/Pinb-D1h* and 4 *Pina-D1a/Pinb-D1a Ha* locus homozygotes. All lines were grown in the field in replicated plots in 2009.

Grain hardness and kernel weight were measured using the Single Kernel Characterization System (SKCS) and protein content by NIR. While *Ha* haplotype was not associated with changes in kernel weight or protein content, it was associated with changes in grain hardness in both recurrent parent populations (Table 8). In the ‘Alpowa’ population, there was a difference of 9.4 units ($P<0.001$), and in the ‘Vanna’ population there was a difference of 12.0 units ($P<0.001$) with the *Pina-D1c/Pinb-D1h* haplotype associated with increased grain hardness in both populations. Overall, seeds with the *Pina-D1c/Pinb-D1h* haplotype were 11.7 units harder than seeds with the *Pina-D1a/Pinb-D1a* haplotype ($P<0.001$).

Table 8. Grain hardness, seed weight and protein content for populations of elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with recurrent parents 'Alpowa' (BC_3F_2; *Pina-D1a/Pinb-D1a*) and 'Vanna' (BC_2F_2 ; *Pina-D1a/Pinb-D1a*) soft white spring wheats.

Haplotype[b]	# of lines	SKCS hardness[c]	Kernel Weight (mg)[c]	Seed Protein (g/kg)[d]
'Alpowa'*4/Elite #14				
Pina-D1a/Pinb-D1a	3	24.4	36.4	116.9
se		1.5	0.7	1.1
Pina-D1c/Pinb-D1h	4	33.8	35.9	114.6
se		1.0	0.9	1
P-value[a]		<0.001	0.642	0.147
'Vanna'*3/Elite #14				
Pina-D1a/Pinb-D1a	4	16.2	36.4	124.3
se		1.1	0.9	1
Pina-D1c/Pinb-D1h	3	28.1	36.4	125.8
se		1.2	1.0	2.2
P-value		<0.001	0.999	0.476
Mean				
Pina-D1a/Pinb-D1a	7	19.7	36.4	121.1
se		1.2	0.6	1
Pina-D1c/Pinb-D1h	7	31.3	36.1	119.4
se		0.9	0.6	1.5
P-value		<0.001	0.72	0.36
Parents				
'Alpowa'	1	28.1	33.4	116.5
se		0.8	2.1	0.1
'Vanna'	1	13.4	28.6	111.3
se		2.3	0.7	0.1
Elite #14	1	37.8	46.8	149.8
se		3.3	2.8	0.2

[a] *P*-values determined using a studentized t-test for SKCS hardness, Kernel weight, seed and flour protein.

[b] *Pina-D1a/Pinb-D1a* is the 'soft type' *Ha* haplotype and *Pina-D1c/Pinb-D1h* is the synthetic *Ha* haplotype.

[c] Kernel hardness and kernel weight were measured using the single kernel characterization system.

[d] Whole grain protein was measured using near infrared reflectance (determined by multiplying nitrogen content by 6.25).

For flour yield, only the ‘Alpowa’ population showed a significant difference, with the *Pina-D1c/Pinb-D1h* lines yielding 1.5% more flour than the ‘soft type’ lines (Table 9). Ash content also trended toward having more in the *Pina-D1c/Pinb-D1h* lines than in the *Pina-D1a/Pinb-D1a* lines, with the differences in the ‘Vanna’ population and the combined means of the two populations being significant (*P*=0.040 and *P*=0.024, respectively). Bran and flour amounts didn’t differ significantly between the two haplotypes for either population. For particle sizes, there are differences that appear to be caused by the *Pina-D1c/Pinb-D1h* haplotype (Table 10). The *Pina-D1a/Pinb-D1a* haplotype had more very small (<53 µm) particles (33.5% vs 30.8% in the ‘Alpowa’ population, 33.8% vs 28.6% in the ‘Vanna’ populations, and 33.7% vs 29.7% overall, *P*=<0.001 in all three cases) and fewer large (>75µm) particles (55.7% vs 58.6% in the ‘Alpowa’ population, 54.9% vs 60.9% in the ‘Vanna’ population, and 55.3% vs 59.6% overall (*P*= <0.001). The lone exception was for particles greater than 150 µm in the ‘Alpowa’ population. These differences in flour particle size distribution point out differences in milling properties conferred by these different haplotypes. There was no significant difference in the amount of flour in the size range between 53 and 75 µm for either of the populations. There were no noticeable trends for the affects of the novel *Ha* locus on SRC and AWRC (Table 11).

Table 9. Mean bran and flour yields after milling and flour ash content of synthetic versus 'soft type' *Ha* locus haplotypes. [Elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with recurrent parents 'Alpowa' (BC_3F_2; *Pina-D1a/Pinb-D1a*) and 'Vanna' (BC_2F_2; *Pina-D1a/Pinb-D1a*).]

Haplotype [b]	% Bran [c]	% Flour yield [c]	Flour Protein (g/kg) [d]	Ash (g) [e]
'Alpowa'*4/Elite #14				
Pina-D1a/Pinb-D1a	31.3	68.8	114	0.4
se	1.1	0.6	4.1	0.0
Pina-D1c/Pinb-D1h	29.7	70.3	118	0.4
se	0.6	0.3	5.2	0.0
P-value [a]	0.144	0.021	0.517	0.201
'Vanna'*3/Elite #14				
Pina-D1a/Pinb-D1a	29.2	70.8	119	0.4
se	0.5	0.3	5.0	0.0
Pina-D1c/Pinb-D1h	28.8	71.3	121	0.5
se	0.7	0.4	6.4	0.0
P-value	0.437	0.476	0.767	0.040
Mean				
Pina-D1a/Pinb-D1a	30.1	70.0	117	0.4
se	0.6	0.4	2.9	0.0
Pina-D1c/Pinb-D1h	29.3	70.7	120	0.5
se	0.5	0.3	3.7	0.0
P-value	0.253	0.037	0.531	0.024
Parents				
'Alpowa'	30.3	69.8	104	0.4
se	2.5	1.8	1.1	0.0
'Vanna'	30.3	69.7	98	0.4
se	0.7	0.3	0.8	0.0
Elite #14	29.8	70.3	145	0.4
se	1.5	0.5	3.1	0.0

[a] *P*-values determined using a studentized t-test.
[b] *Pina-D1a/Pinb-D1a* is the 'soft type' *Ha* haplotype and *Pina-D1c/Pinb-D1h* is the synthetic *Ha* haplotype.
[c] Bran and flour were weighed after milling but prior to sifting.
[d] Flour protein measured with NIR (determined by multiplying nitrogen content by 6.25).
[e] Flour ash was estimated using near infrared reflectance and calculated as number of grams of ash in 100 g of flour.

Table 10. Flour particle size distribution of synthetic versus 'soft type' *Ha* locus haplotypes. [Elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with recurrent parents 'Alpowa' (BC_3F_2; *Pina-D1a/Pinb-D1a*) and 'Vanna' (BC_2F_2 ; *Pina-D1a/Pinb-D1a*).]

Haplotype [b]	% >150 µm [c]	% <150 - >75 [d]	%<75 - >53 [e]	% <53 [f]
'Alpowa'*4/Elite #14				
Pina-D1a/Pinb-D1a	26.3	29.4	10.8	33.5
se	0.3	0.3	0.3	0.4
Pina-D1c/Pinb-D1h	26.5	32.1	10.6	30.8
se	0.3	0.2	0.2	0.5
P-value [a]	0.596	<0.001	0.429	<0.001
'Vanna'*3/Elite #14				
Pina-D1a/Pinb-D1a	24.2	30.7	10.5	33.8
se	0.3	0.4	0.7	0.5
Pina-D1c/Pinb-D1h	26.3	34.6	10.5	28.6
se	0.4	0.4	0.3	0.5
P-value	<0.001	<0.001	0.941	<0.001
Mean				
Pina-D1a/Pinb-D1a	25.1	30.2	10.7	33.7
se	0.3	0.3	0.2	0.4
Pina-D1c/Pinb-D1h	26.4	33.2	10.5	29.9
se	0.2	0.3	0.2	0.4
P-value	0.006	<0.001	0.631	<0.001
Parents				
'Alpowa'	27.0	30.1	11.6	32.0
se	0.8	0.5	0.5	1.1
'Vanna'	21.1	26.8	14.3	37.9
se	0.2	0.6	0.7	0.6
Elite #14	32.5	38.3	9.9	19.3
se	0.6	0.9	0.2	1.01

[a] *P*-values determined using a studentized t-test.
[b] *Pina-D1a/Pinb-D1a* is the 'soft type' *Ha* haplotype and *Pina-D1c/Pinb-D1h* is the synthetic *Ha* haplotype.
[c] Percent of flour particles larger than 150 µm.
[d] Percent of flour particles between 150 µm and 75 µm.
[e] Percent of flour particles between 75 µm and 53 µm.
[f] Percent of flour particles smaller than 53 µm.

Table 11. Mean solvent retention capacity percentages for the *Pina-D1a/Pinb-D1a* 'soft type' *Ha* locus haplotype compared to the *Pina-D1c/Pinb-D1h* synthetic *Ha* locus haplotype. [Elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with recurrent parents 'Alpowa' (BC_3F_2; *Pina-D1a/Pinb-D1a*) and 'Vanna' (BC_2F_2 ; *Pina-D1a/Pinb-D1a*).]

	Solvent Retention Capacity				
Haplotype [b]	% Sucrose [c]	% Sodium Carbonate [c]	% Alkaline Water [d]	% Lactic Acid [c]	% Water [c]
Alpowa*4/Elite #14					
Pina-D1a/Pinb-D1a	101.2	89.8	76.6	152.6	72.8
se	0.8	2.1	1.2	2.2	0.8
Pina-D1c/Pinb-D1h	101.0	87.2	75.2	149.2	73.5
se	0.6	1.6	0.6	2.3	0.4
P-value [a]	0.867	0.317	0.256	0.305	0.374
Vanna*3/Elite #14					
Pina-D1a/Pinb-D1a	97.8	82.1	72.8	119.8	68.2
se	0.9	1.2	0.8	3.0	1.1
Pina-D1c/Pinb-D1h	97.4	79.5	71.0	122.3	66.5
se	0.8	1.4	0.7	4.4	0.7
P-value	0.722	0.157	0.115	0.624	0.222
Mean					
Pina-D1a/Pinb-D1a	99.5	85.4	74.4	133.8	70.2
se	0.8	1.3	0.8	3.7	0.8
Pina-D1c/Pinb-D1h	99.2	83.9	73.4	137.7	70.5
se	0.7	1.3	0.6	3.4	0.7
P-value	0.773	0.414	0.289	0.448	0.758
Parents					
Alpowa	93.8	90.0	80.3	150.6	73.4
se	10.1	2.8	0.6	1.9	0.9
Vanna	103.4	85.7	76.5	125.7	71.8
se	1.9	3.4	0.6	4.0	0.6
Elite #14	108.9	76.8	70.3	103.4	66.6
se	3.7	1.1	1.17	2.8	0.9

[a] *P*-values determined using a studentized t-test.
[b] *Pina-D1a/Pinb-D1a* is the 'soft type' *Ha* haplotype and *Pina-D1c/Pinb-D1h* is the synthetic *Ha* haplotype.
[c] Determined using AACC method 56-11. Calculated as the amount of solvent retained by the flour as a percentage of the original flour weight at a 14% flour moisture level.
[d] Determined using AACC method 56-10. Calculated as the amount of solvent retained by the flour as a percentage of the original flour weight at a 14% flour moisture level.

Cookie quality was also impacted by *Ha* locus associated differences (Table 12). Cookie diameter was smaller with the *Pina-D1c/Pinb-D1h* synthetic *Ha* locus haplotype in the 'Vanna' populations and overall ($P<0.001$ for the 'Vanna' population; and $P=0.020$ for the two combined). Cookie height was greater for the *Pina-D1c/Pinb-D1h* haplotype for the Vanna population and when combined over the two populations ($P=0.040$ for the 'Vanna' population; and $P=0.034$ for the two combined). Cookie weight loss and top grain score showed no significant differences between the differing *Ha* loci (Table 12 and Fig.4).

Table 12. Baking quality data represented as means comparing the *Pina-D1a/Pinb-D1a* 'soft type' haplotype to the *Pina-D1c/Pinb-D1h* synthetic haplotype. [Elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with recurrent parents 'Alpowa' (BC_3F_2; *Pina-D1a/Pinb-D1a*) and 'Vanna' (BC_2F_2 ; *Pina-D1a/Pinb-D1a*).]

Haplotype [b]	Diameter (cm) [c]	Weight loss (g) [d]	Cookie height (cm) [c]	Top grain score (0-9) [e]
Alpowa*3/Elite #14				
Pina-D1a/Pinb-D1a	7.98	4.6	0.90	2.4
se	0.0	0.0	0.0	0.3
Pina-D1c/Pinb-D1h	7.94	4.4	0.93	3.2
se	0.0	0.1	0.0	0.3
P-value [a]	0.592	0.064	0.293	0.269
Vanna*2/Elite #14				
Pina-D1a/Pinb-D1a	8.25	4.9	0.87	3.0
se	0.0	0.1	0.0	0.4
Pina-D1c/Pinb-D1h	8.04	4.7	0.93	2.1
se	0.0	0.1	0.0	0.4
P-value	<0.001	0.239	0.040	0.808
Mean				
Pina-D1a/Pinb-D1a	8.11	4.8	0.89	2.9
se	0.0	0.1	0.0	0.2
Pina-D1c/Pinb-D1h	8.00	4.5	0.93	3.1
se	0.0	0.1	0.0	0.3
P-value	0.020	0.040	0.034	0.522

(Table 12 continued)

Parents				
Alpowa'	8.02	5.2	0.89	2.8
se	0.0	0.2	0.0	0.4
Vanna'	8.18	4.7	0.92	3.8
se	0.0	0.1	0.0	0.6
Elite #14	8.04	4.5	0.91	2.1
se	0.1	0.2	0.0	0.3

[a] *P*-values determined using a studentized t-test.
[b] *Pina-D1a/Pinb-D1a* is the 'soft type' *Ha* haplotype and *Pina-D1c/Pinb-D1h* is the synthetic *Ha* haplotype.
[c] Cookie diameter and cookie height is measured using two cookies made with the same flour side by side or one on top of the other and dividing by 2.
[d] Weight loss was calculated by weighing the cookies on the pan before and after they were baked and finding the difference.
[e] Top grain score is determined through the use of a standardized set of cookies with 9 being the best.

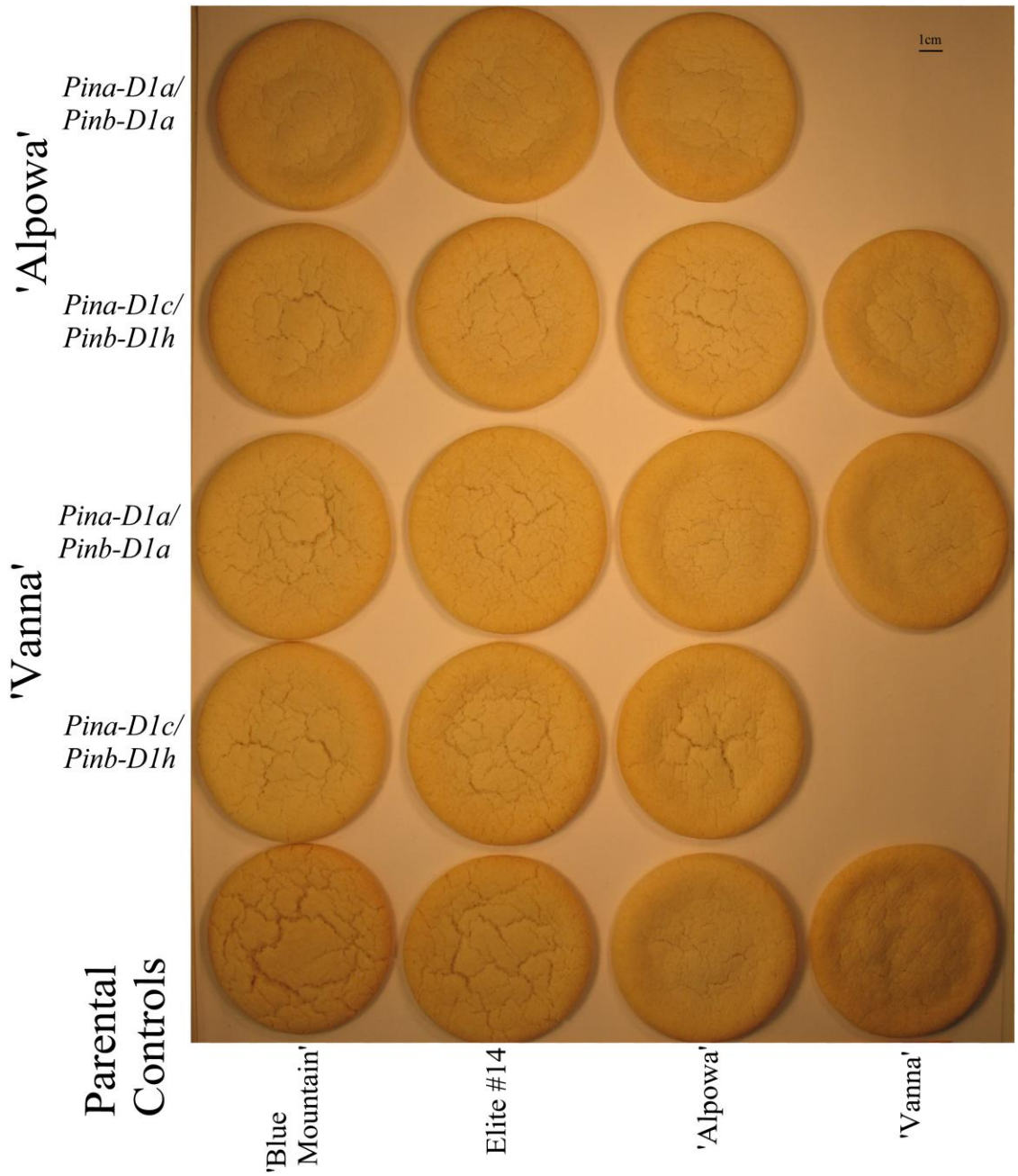

Figure 4. Picture of the top of the two flour controls ‘Blue Mountain’, Elite line 14, along with three cookies from the ‘Alpowa’ population and three cookies form the ‘Vanna’ population. Each population is represented by a ‘soft type’ parent, one sample with the synthetic *Ha* locus haplotype and one sample with the ‘soft type’ *Ha* locus haplotype. ‘Blue Mountain’ is used as a control with known cookie baking quality and is given a top grain score of 6. [Elite synthetic line #14 (*Pina-D1c/Pinb-D1h*) cross with recurrent parents ‘Alpowa’ (BC_3F_2; *Pina-D1a/Pinb-D1a*) and ‘Vanna’ (BC_2F_2 ; *Pina-D1a/Pinb-D1a*).]

Discussion

The objective of this study was to determine the milling and baking quality effects associated with the *Pina-D1c/Pinb-D1h Ha* locus haplotype, which confers a harder grain phenotype than the *Pina-D1a/Pinb-D1a* haplotype (Reynolds et al. 2009). This was done by introgressing the *Ha* haplotype *Pina-D1c/Pinb-D1h* into the soft white spring wheat cultivars 'Alpowa' and 'Vanna'. Previously, these populations have been tested for grain hardness, showing an average of an 10.6 SKCS unit increase (Table 8), yet showed no differences in the expression of PIN proteins or RNA transcripts (Chapter 1). Logically, the next step was to test whether the *Pina-D1c/Pinb-D1h Ha* haplotype is associated with changes in soft wheat milling and baking quality.

The first characteristics that were analyzed were milling and flour properties. Flour protein was measured in order to better predict how these flours would bake into cookies, and no significant difference was found for this characteristic. Flour ash does show an increase in the *Pina-D1c/Pinb-D1h* haplotype (Table 9). Flour ash is used as an indicator of flour grade color (Symons and Dexter, 1991), but more importantly for this study it correlates with grain hardness, as shown in lines with extremely low ash content as a result of transgenic overexpression of puroindolines (Hogg et al. 2005). Here, overall flour yield is affected only in the 'Alpowa' population (Table 9), but it is clear that there is a greater proportion of smaller particles for the *Pina-D1a/Pinb-D1a* than the *Pina-D1c/Pinb-D1h* haplotype in both the 'Alpowa' and the 'Vanna' populations (Table 10). This agrees with previous findings that softer grain texture results in smaller particle size (Gaines, 1985). Smaller particle size often result in more water absorption, either caused

by increased starch damage during particle size reduction (Yamazaki, 1959), or by increased surface area (Gaines, 1985). The sodium carbonate retention capacity and alkaline water retention tests can give insight into the amount of flour starch damage and can be used to predict end product quality differences (Gaines, 2000; Slade and Levine, 1994). Here, both tests indicate that the two tested *Ha* loci do not affect solvent retention capacities (Table 11), which would mean that starch damage is not a factor in the smaller particle size, and will not be a factor to consider in any other end product quality differences. This could be explained by the fact that soft wheats suffer from less starch damage than hard wheats (Slaughter et al. 1992) and, therefore, the slight differences in hardness seen between the *Pina-D1a/Pinb-D1a* and the *Pina-D1c/Pinb-D1h* represent a smaller range than between hard and soft wheat classes. Sucrose retention is indicative of pentosan and gliadin impact on the absorption of water and, along with protein content, is highly correlated with sugar snap cookie diameter (Gaines, 2000; Guttieri et al. 2001; Guttieri and Souza, 2003; Roccia et al. 2006). Sucrose retention capacity has also been shown to be correlated with sodium carbonate retention capacity (Guttieri et al. 2001). Here, sucrose retention was unaffected by *Ha* haplotype (Table 11), which would indicate that pentosans and/or gliadins are not responsible for increased water absorption. Lactic acid retention points to glutenins as the reason for increased water absorption (Gaines, 2000). Here, lactic acid retention is also unaffected by the *Pina-D1c/Pinb-D1h* haplotype, but there is clearly a difference between the ‘Alpowa’ and ‘Vanna’ populations. Finding an increase like this is not surprising because ‘Alpowa’ is known to have the 5+10 subunits of the high molecular weight glutenin genes, while ‘Vanna’ does not (Park et al.

2003). Therefore, the differences seen in lactic acid retention are not a product of the *Ha* locus, which is what is being tested here.

Water absorption has an underlying effect on the baking quality of wheat flour. As explained above, the water absorption in these samples could be caused by differences in particle size or starch damage, or by the presence of pentosans, gliadins, or glutenins. Upon baking sugar snap cookies from the flour, cookie diameter was higher in the *Pina-D1a/Pinb-D1a* haplotype (Table 12). In contrast, cookie height was smaller in the *Pina-D1a/Pinb-D1a Ha* haplotype. High levels of water absorption reduce the amount of spreading when cookies are baked (Gaines, 2000). The differences seen here could be explained by the low level of variability, fractions of a millimeter, in the measurements. It appears that all genotypes have a high degree of water absorption which would lead to their relatively poor top grain scores (Figure 4). An interesting aspect of the cookie baking was the differences between the 'Alpowa' and 'Vanna' populations. Again, these are most likely caused by the 5+10 subunits of the high molecular weight glutenins in 'Alpowa', which would increase water absorption and decrease cookie spread and top grain score.

Conclusion

Here, we have demonstrated that the novel *Ha* locus *Pina-D1c/Pinb-D1h* confers larger flour particle size, which leads to smaller sugar snap cookies. The data shown here would suggest that breeders would not be interested in this novel haplotype because there is no improvement in baking quality. It is possible, however, that this minor change in

end product quality be beneficial in breeding programs. If these lines were grown in a more appropriate environment, it would be possible that there is a more significant difference in cookie spread and top grain score than we see here. These findings are also helpful in the further understanding of the function of the Pin genes in the plant overall.

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APPENDIX A

BACKCROSS POPULATION AGRONOMIC DATA

Haplotype	Plant Height (cm) [a]	Yield (kg/row) [a,b]	Flowering Time (days) [c]
Alpowa*4/Elite #14			
Pina-D1a/Pinb-D1a	83	581	67.83
se	0.9	1.8	0.30
Pina-D1c/Pinb-D1h	84	562	68.14
se	0.3	3.4	0.21
P-value	0.531	0.641	0.391
Vanna*3/Elite #14			
Pina-D1a/Pinb-D1a	83	549	68.79
se	1.0	6.3	0.53
Pina-D1c/Pinb-D1h	84	625	69.09
se	2.1	2.2	0.56
P-value	0.334	0.316	0.724

[a] Plant height, row length, and yield were measured in 2009 only due to hail in 2008.
[b] Yield is the average grain from two row plots.
[c] Flowering time was measured in 2008 and is reported as the number of days between planting and anthesis.