PUROINDOLINES: THEIR CONTROL OVER WHEAT GRAIN HARDNESS AND INFLUENCE ON MILLING AND BREAD BAKING TRAITS.

by

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ABSTRACT

Wheat grain is sold based upon several physiochemical characteristics, one of the most important being grain texture. Grain texture in wheat directly affects many end use qualities such as milling yield, break flour yield, and starch damage. The Hardness *(Ha)* locus located on the short arm of chromosome 5D is known to control grain hardness in wheat. This locus contains the puroindoline a (*pina*) and puroindoline b (*pinb*) genes. All wheats to date that have mutations in *pina* or *pinb* are hard textured, while wheats possessing both the 'soft type' *pina-D1a* and *pinb-D1a* sequences are soft. Furthermore, it has been shown that complementation of the *pinb-D1b* mutation in hard spring wheat can restore a soft phenotype. Here, the first objective was to identify and characterize the effect the puroindolines have on grain texture independently and together. The second objective was to determine which milling bread baking characteristics are affected by the seed specific over-expression of puroindolines. To accomplish both of these objectives a hard red spring wheat, possessing the *pinb-D1b* mutation, was transformed with 'soft type' *pina* and *pinb*, creating transgenic isolines that have added *pina*, *pinb*, or *pina* and *pinb*. Northern blot analysis of developing control and transgenic lines indicated that grain hardness differences were correlated with the timing of the expression of the native and transgenically added puroindolines. The addition of PINA decreased grain hardness less than the reduction seen with added PINB. Friabilin abundance was correlated with the presence of both 'soft type' PINA and PINB and did not correlate well with total puroindoline abundance. Selected T_3 lines were grown in a field trial with two replications under dry and irrigated field conditions. Harvested grain was then milled and baked. Lines transformed with the puroindolines exhibited decreased total flour yields and increased break flour yields, yielding flour with lower protein and ash content. Decreases in loaf volume, mixograph absorption, and crumb grain scores were also observed in transgenic lines. These results demonstrate that the puroindolines can be used to profoundly influence grain hardness and a variety of milling and bread baking traits in wheat.

CHAPTER 1

WHEAT PUROINDOLINES INTERACT TO FORM FRIABILIN AND CONTROL WHEAT GRAIN HARDNESS

Introduction

Wheat (*Triticum aestivum* L.) is one of the world's most important food crops and is traded across the globe based in part on the physical characteristic of grain hardness. Based on this, wheat is typically divided into hard and soft classes. Grain hardness in wheat is also a main determinant in end product quality. Soft wheat requires less energy to mill, yields smaller flour particles with less starch damage, and absorbs less water compared to hard wheat (Symes 1965; Symes 1969). Soft wheats are generally used to make cookies and pastries while hard wheats are typically used to make breads (Reviewed in Morris and Rose, 1996).

Wheat grain hardness is controlled by the Hardness locus (*Ha*) (Symes 1965; Baker 1977), which is located on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). Greenwell and Schofield (1986) found friabilin could be used as a marker protein for grain hardness. They reported that friabilin is an abundant 15 kDa protein on water-washed starch granules from soft wheat, and that little to no friabilin is found on water-washed starch granules from hard wheat (Greenwell & Schofield 1986). It was later shown that genes encoding for friabilin were closely linked to the *Ha* locus (Jolly et al. 1993; Jolly et al. 1996). N-terminal sequencing of friabilin revealed that it is composed mainly of two proteins, puroindoline A (PINA) and puroindoline B (PINB)

(Jolly et al. 1993; Morris et al. 1994). These puroindoline proteins contain a unique tryptophan rich domain that is thought to be involved in the binding of phospholipids on the surface of starch granules (Gautier et al. 1994; Marion et al. 1994). Located within the *Ha* locus are the tightly linked genes puroindoline a (*pina)* and puroindoline b (*pinb)* (Sourdille et al. 1996; Giroux and Morris 1997; Tranquilli et al. 1999; Turnbull et al. 2003) which have been cloned and encode for their respective proteins described above (Gautier et al. 1994).

To date all hard wheats characterized carry a mutation in one of their puroindoline genes, with the known mutations being single nucleotide changes in the coding sequence of *pinb* and a *pina* null mutation (Giroux & Morris 1998; Lillemo and Morris 2000; Morris et al 2001). All soft wheats examined so far carry identical *pina-D1a* and *pinb-D1a* sequences (Giroux & Morris 1998; Lillemo and Morris 2000; Morris et al. 2001). Because of the tight association between the *Ha* locus, friabilin, and the puroindolines (Sourdille et al. 1996; Giroux and Morris 1997) it has been suggested that the puroindolines are the primary genetic elements for controlling grain hardness in wheat (Giroux and Morris 1998). To investigate this hypothesis, rice, which has no homologues to the puroindolines (Gautier et al. 2000), was transformed with 'soft type' *pina* and *pinb* (Krishnamurthy and Giroux 2001). The result was a decrease in rice kernel hardness, starch damage, and particle size. Further supporting evidence for the role of puroindolines on grain hardness was that the transgenic complementation of *pinb* in the hard spring wheat variety 'Hi-Line', which carries the *pinb-D1b* variant sequence, produced a soft wheat phenotype (Beecher et al. 2002). The 'soft type' *pinb* transgenic

lines had increased levels of friabilin, decreased grain hardness, and starch granules that resembled those indicative of soft wheats first described by Barlow (1973). Here we have created six unique transgenic lines in the Hi-Line background that have the addition of 'soft type' *pina*, *pinb*, or *pina* and *pinb* to investigate the role of the puroindolines in controlling wheat grain hardness.

Materials and Methods

Plant Material and Growth Conditions

In this study the hard spring wheat cultivar Hi-Line (Lanning et al. 1992) was used for transformation. Hi-Line carries the 'soft type' *pina* sequence, *pina-D1a*, and the variant *pinb* sequence, *pinb-D1b*, which contains a glycine to serine substitution at the 46th residue of PINB (Giroux et al. 2000). 'Heron' (Giroux and Morris 1998) is a soft, white wheat cultivar that carries the 'soft type' *pina-D1a* and *pinb-D1a* sequences. Plants were grown in a greenhouse at the Montana State University-Bozeman Plant Growth Center in eight-inch pots, two plants per pot, with four plants per replication. The temperature targets were 22˚C during the day and 14˚C at night. Supplemental lighting was provided during the hours of 5 a.m. to 9 a.m., and 4 p.m. to 9 p.m. by 1,000 W metal halide lamps that generated 400-µE $m^{-2}s^{-1.}$ Plants were watered as needed with 0.25 g of Peters 20-20-20 General Purpose N-P-K plant food per liter of water. Plants used for northern blot analysis, protein analysis, and particle size index were grown at the Montana State University-Bozeman Arthur H. Post Field Research Farm during the summer of 2002 under irrigated conditions.

Constructs

The *pina* expression vector, pGA1.8 (Figure 1), was created in our laboratory using the untranslated glutenin gene flanking sequences from pGlu10H5 (Blechl and Anderson 1996). The primers ABH5 (5´CG*GGATCC*AACAATGAAGGCCCTCTTCCTCATAGG 3´) and AXP3 (5´ AACTGCAG*TCTAGA*TCATCACCAGTAATAGCCAATA 3´) were used to amplify the 'soft type' *pina* sequence, *pina-D1a,* from a 'Chinese Spring' genomic DNA extract (Reid and Anderson 1996) using *Taq* DNA polymerase (Promega, Madison, WI). These primers included the addition of *BamHI* and *XbaI* sequences which are italicized above. The cycling parameters consisted of an initial denaturation step at 94ºC for three min., followed by 40 cycles of 94ºC for 45s, 55ºC for 30s, 72ºC for 90s, and a final extension at 72ºC for five min. The amplified product was digested with *BamHI* and *XbaI*, and then ligated between the glutenin flanking sequences, *Dy10* and *Dx5*, of pGlu10H5 (Blechl and Anderson 1996). The result was the complete replacement of the glutenin coding sequence with that of the 'soft type' *pina* coding sequence. The 'soft type' *pina* coding sequence and its flanking glutenin sequences were then placed into a modified pET28a backbone (Invitrogen, San Diego, CA).

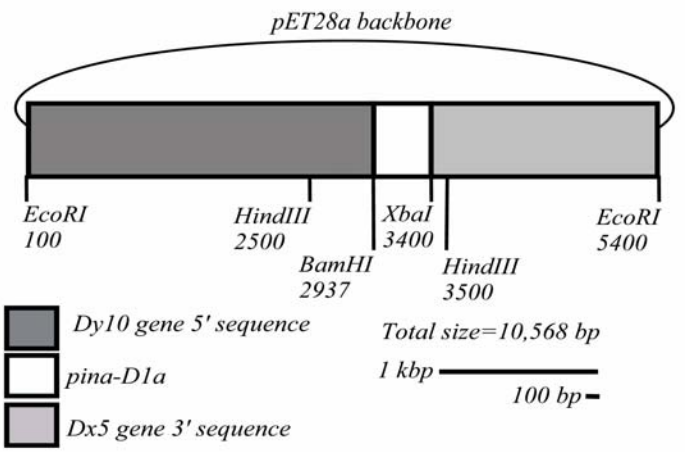

Figure 1. Above is a schematic of the *pina* expression vector, pGA1.8. The glutenin coding region of the pGlu10(5) construct has been replaced with the 'soft-type' *pina* coding sequence, which was amplified from Chinese Spring genomic DNA. The 'soft type' *pina* sequence is under the control of the glutenin regulatory elements *Dy10* (5′) and *Dx5* (3′).

The *pinb* expression vector, pGB4.20, was constructed as described by Beecher et al. (2002) and the *Bar* expression vector pRQ101A used for selection has been described previously (Sivamani et al. 2000). The *Bar* construct confers resistance to the herbicides bialaphos (Meiji Seika Kaisha Ltd, Japan) and glufosinate (AgrEvo USA Company, Willmington, DE).

Transformation of Wheat With *Pina* and *Pinb* and Regeneration

Immature embryos from the cultivar Hi-Line were transformed and regenerated as described by Beecher et al. (2002). Callus tissue was bombarded with pGA1.8, pGB4.20, and pRQ101A construct DNA in a 2.5:2.5:1 molar ratio respectively to obtain HGAB lines with added pGA1.8 and pGB4.20 and with a 5:1 molar ratio of pGA1.8:pRQ101A to obtain HGA lines with added pGA1.8. The HGB lines with added pGB4.20 have been described previously (Beecher et al. 2002).

PCR, Herbicide Screen, and Single Plant PIN Protein Analysis

Recovered T_0 plants were PCR screened to determine if they carried the transgene(s). Genomic DNA was extracted from young leaves (Riede and Anderson 1996) and two different PCR reactions were performed. To identify the presence of pGA1.8 the primers PA5 (5´ ATGAAGGCCCTCTTCCTCA 3´) (Gautier et al. 1994), which hybridizes to the 5´ end of the 'soft type' *pina* coding sequence, and PGUNIV (5´CTAAAGTGCATGCATGCC3´), which hybridizes to the 5´ end of *Dx5*, were used. To identify the presence of pGB4.20 the primers PB5 (5´ATGAAGACCTTATTCCTCCTA3´) (Gautier et al. 1994), which hybridizes to the 5´ end of the 'soft type' *pinb* coding sequence, and PGUNIV (described above) were used. The temperature regime used for PCR was the same as described above. The presence of both pGA1.8 and pGB4.20 is indicated by a 495 base-pair PCR product. *Bar* presence and expression in these T_0 plants was checked using the leaf paint method. This was accomplished by applying 0.1% glufosinate (AgrEvo USA Company, Willmington, DE) to individual leaves of plants using a cotton swab, which were then scored as either resistant or susceptible after seven days. Leaves of susceptible plants were chlorotic while resistant plants remained green. In the T_1 generation homozygous plants were identified using the PCR and leaf paint methods where 12 or more consecutive T_2 progeny from an individual T_1 plant were positive for both the pin transgene(s) and herbicide resistance. Analysis of puroindoline protein content in 12 or more T_2 individual kernels of T_1 progeny plants was also used to confirm homozygosity in the T_1 generation. In all lines, the puroindoline transgene co-segregated with *Bar*.

Herbicide Test

Homozygous T_2 seed lines (homozygous for the *Bar* and puroindoline transgenes) were grown in the greenhouse or field and T_3 progeny were re-screened for homozygosity with a herbicide test. Approximately 24 T_3 seeds were planted in 13 cm^2 six packs, four seeds per pack. Plants were sprayed with 0.1% glufosinate (AgrEvo USA Company, Wilmington, DE) during the two-leaf stage of growth. After seven days plants were scored as being either resistant or susceptible. Susceptible plants were characterized by wilting, systemic chlorosis, and localized necrosis, while resistant plants remained green.

Northern Blot Analysis

To obtain RNA from immature wheat kernels heads were collected from the field at 21 days post anthesis (DPA), frozen in liquid N_2, and stored at –80˚C. For the twelve samples in the developmental series additional heads were collected at 7, 14, and 28 DPA. RNA was extracted from immature kernels using a standard Trizol protocol (Invitrogen, San Diego, CA) with the following changes.

First, seed powder was obtained by grinding the samples with a mortar and pestle in liquid N_2. Approximately 0.2 cm^3 of seed powder was placed in a 2 mL tube and then 0.5 mL of RNA extraction buffer (50 mM Tris, pH 9.0, 200 mM NaCl, 1.0% Sarcosyl, 20 mM EDTA, 5 mM DTT, and H_2O) was added and the sample was vortexed until homogenous. Next, 0.5 mL of phenol/chloroform/isoamyl alcohol (49:49:2) was added, the samples were vortexed until homogenous, and centrifuged for five min. at 13,000 x g, 4˚C. Then 0.5 mL of the upper aqueous layer was removed and placed into a 2 mL tube

containing 1 mL of Trizol reagent, which was then vortexed. Next, 0.2 mL of chloroform was added, vortexed until homogenous, and then centrifuged for five min. at 13,000 x g, 4˚C. Then 0.75 mL of the upper aqueous layer was removed and placed in a 2 mL tube, followed by adding 0.5 mL of chloroform, vortexing, and centrifuging for five min. at 13,000 x g, 4˚C. Next, 0.6 mL of upper aqueous layer was removed and placed in a 2 mL tube containing 1.2 mL 100% ethanol and 60 µL of 3 M sodium acetate, vortexed, and then left to precipitate for one hour at -80˚C. Samples were then centrifuged for 20 min. at 13,000 x g, 4˚C, and RNA pellets were washed with 1 mL of 70% ethanol. RNA pellets were re-suspended in 50 µL of TE (10 mM Tris, 1 mM EDTA pH 8.0) and RNA concentration determined using a spectrophotometer.

Northern blot analysis was performed using standard methods previously described (Giroux and Morris 1997). One microgram of RNA was fractionated on a formaldehyde agarose gel and then blotted onto a nylon membrane (Osmonics, Inc., Minnetonka, MN). The blots were then hybridized, washed, and exposed to film as previously described (Beecher et al. 2002). The templates used to make the probes for *pina* and *pinb* were Hi-Line genomic DNA PCR products. The primers used to make the probe template for pGA1.8 were PA5 (described above) and PA3 (5´TCACCAGTAATAGCCAATAGTG3´) (Gautier et al. 1994), which hybridizes to the 3´ end of the ‘soft type’ *pina* coding sequence. The probe template for pGB4.20 was made using the primers PB5 (described above) and PB3 (5´TCACCAGTAATAGCCACTAGGGAA3´) (Gautier et al. 1994), which hybridizes to

the 3´ end of the ‘soft type’ *pinb* coding sequence. To quantify total *pina* and *pinb* an expression scale was devised that included 0.5X, 1X, 2X, 4X, and 6X total RNA concentration levels, where 1X equaled 1 µg of total RNA. The RNA used to make this scale was taken from the Hi-Line total RNA sample.

TX-114 Protein Extraction and Analysis

The puroindoline proteins were extracted following methods previously described by Giroux et al. (2003). To quantify total puroindoline abundance a scale ranging from 1X to 8X, in multiples of 1X, was used. This scale was constructed using a Hi-Line TX-114 protein extract where 1X equaled 5 µL.

Friabilin Protein Extraction and Analysis

Friabilin was isolated from the surface of starch granules by a modification of previously described methods (Sulaiman and Morris 1990; Bettge et al. 1995). Samples were milled using a KT-3303 (Perten, Springfield, IL) laboratory mill. For each sample 100 mg of whole-wheat flour was steeped for 30 min. in a 1.5 mL microfuge tube containing 0.5 mL of 0.1 M NaCl. Using a microdrill and a micropestle the flour was worked into a ‘dough ball’ consisting of gluten and bran. The dough ball was pushed to the bottom of the tube and the aqueous starch solution was transferred to the top of a 1.5 mL microfuge tube containing 1 mL of 80% cesium chloride. The starch water suspension and CsCl were then centrifuged at 13,000 x g for three min. The CsCl was decanted and the starch was washed with 1 mL water. The starch water solution was vortexed until homogenous and then centrifuged for three min. at 13,000 x g. After

centrifugation the water was decanted and the starch was water-washed two more times. The starch was then dried using acetone. After removing the acetone and letting the starch dry, the starch was weighed to ensure equal loading of samples. To the dried starch 200 µL of 50% isopropanol, 0.5 M NaCl was added. The samples were then vortexed and left to incubate at room temperature for 30 min. After incubation the samples were centrifuged for three min. at 13,000 x g and the supernatant was transferred to a 1.5 mL tube. Next, 120 µL of cold acetone was added to the supernatant, vortexed, and then samples were left to incubate overnight at -20ºC. Samples were then removed from -20ºC, centrifuged for three min. at 13, 000 x g, and the supernatant was removed. To the pellet 400 µL of -20ºC acetone was added, followed by vortexing and another overnight incubation at -20ºC. The following day the samples were removed from the -20ºC and centrifuged as described above. Next, the acetone was removed followed by an acetone wash to dry the pellet. After the pellet was completely dry the correct amount of standard SDS sample buffer was added based on the starch weight from above (120 µL buffer/100 mg sample).

Samples were then heated at 70ºC for ten min., followed by a brief vortexing. The extracted proteins were then fractionated using SDS-PAGE on 10%-20% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA). Gels were then stained, de-stained, and dried as described in Giroux et al. (2003). To quantify the amount of bound puroindoline a scale ranging from 1X to 8X, in multiples of 1X was used. This scale was constructed using a Hi-Line TX-114 protein extract where 1X equaled 5 µL.

Grain Hardness Measurement and Particle Size Analysis

Four replications of six transgenic lines, along with Hi-Line and 161 were grown in a greenhouse arranged in a randomized block design. An experimental unit was a single pot with four plants. To determine grain hardness both the Single Kernel Characterization System, SKCS 4100 (Perten Instruments, Springfield, IL), and particle size index were used. SKCS analysis was performed on seed from the replicated greenhouse study described above while particle size index was performed on a single replication of grain collected from the 2002 field study. Seeds used in the developmental study were obtained by harvesting three heads for each line from the field, which were then air-dried for seven days. After seven days the heads were thrashed and the seeds were air-dried for an additional day. Seeds were then left to equilibrate for another week before being analyzed. Particle size index was determined following previously described methods [AACC (2000) Method 55-30] with the following changes. Approximately 10 g of whole-wheat flour was sifted through a 90 μm sieve for ten min. Particle size index reported is the percentage of flour recovered that smaller than 90 μm.

Results

Analysis of Transgenic Wheat Plants

Using biolistic transformation seven unique T_0 lines were created in the Hi-Line background (Table 1). HGB-19 and HGB-12 were PCR positive for only the 'soft type' *pinb* transgene, HGA-3 and HGA-1 were positive for the 'soft type' *pina* transgene only, while HGAB-2 and HGAB-3 were positive for both 'soft type' *pina* and *pinb* transgenes.

161 was transformed with only the pRQ101A construct and is PCR negative for the puroindoline transgenes as is the untransformed line Hi-Line. Homozygous PCR positive plants were selected in the T_1 generation based on SKCS scores, individual plant PIN protein levels, and transgene presence in T_1 progeny. Homozygous T_2 lines (*Bar* and puroindoline transgenes) were then planted at the Montana State University-Bozeman Arthur H. Post Field Research Farm. After harvesting the T_2 generation approximately 25 T_3 plants from each of the eight lines was tested to confirm homozygosity, transgene stability, and transgene heritability using a herbicide screen (Table 1) and SKCS.

Table 1. T_0 PCR data for pGA1.8 and pGB4.20 and T_3 herbicide test for selected lines.

Line[a]	PCR[b]		Herbicide test[c]	TX-114 test[d]
	pGA1.8	pGB4.20	resistant/susceptible	*pina /pinb*
Hi-line	-	-	0/24	0/0
161	-	-	23/0	0/0
HGB-19	-	+	27/0	0/12
HGB-12	-	+	26/0	0/12
HGAB-2	+	+	24/0	12/12
HGAB-3	+	+	0/25	12/12
HGA-3	+	-	24/0	12/0
HGA-1	+	-	11/13	6/0

a- All lines were derived from the spring wheat variety Hi-Line. All T_3 lines were derived from the spring wheat variety Hi-Line, which carries a 'soft type' *pina-D1a* sequence and a mutated *pinb-D1b* sequence.
b- PCR screening was performed on genomic DNA extracts from T_0 plants using primers specific for pGA1.8 and pGB4.20
c- T_3 plants were sprayed with 0.1% glufosinate and scored seven days later as either resistant or susceptible to the herbicide.
d- To determine homozygosity, 12 individual T_2 seeds from each line were scored using TX-114 protein samples.

The untransformed Hi-Line progeny were all susceptible while all of the 161 progeny tested were resistant to the herbicide glufosinate (AgrEvo USA Company, Willmington, DE). Both of the HGB lines, HGAB-2, and HGA-3 had 100% herbicide

resistant T_3 progeny. Only 46% of HGA-1 progeny and none of the HGAB-3 progeny showed glufosinate resistance. HGAB-3 was homozygous for both the *pina* and *pinb* transgenes based upon PCR results of 12 or more consecutive T_2 and T_3 progeny. Based on these herbicide and *pin* transgene test results all lines were homozygous except for HGA-1 which is a heterozygote for both *Bar* and the *pina* transgene.

Increased Transcript Levels of *Pina* and *Pinb*

Total RNA was extracted from developing wheat kernels at 21 DPA and analyzed (Figure 2). The two control lines Hi-Line and 161 do not possess either pGB4.20 or pGA1.8 but show transcript accumulation of the native *pina-D1a* and *pinb-D1b* sequences. HGAB-3 has the highest increase in transcript accumulation with over a six-fold increase in *pina* transcript levels and a four-fold increase in *pinb* transcript levels. HGAB-2 has a five-fold increase in *pina* transcript levels and a three-fold increase in *pinb* transcript levels. Both of the HGB lines have a four to five-fold increase in *pinb* transcript levels, while HGB-12 has *pina* transcript levels equal to that observed in Hi-Line and HGB-19 has slightly elevated *pina* transcript levels valued at 1.5. The HGA lines have *pinb* transcript levels equal to that seen in Hi-Line, but HGA-3 has a five-fold increase in the transcript levels of *pina* and HGA-1 has a three-fold increase in *pina* transcript levels (Table 2).

Table 2. Puroindoline expression levels, total puroindoline content, and bound puroindoline content (friabilin) for selected lines

Line	*pina*[a]	*pinb*[a]	'soft' PINA[b]	'soft' PINB[b]	total 'soft' PIN[c]	friabilin PINA[d]	friabilin PINB[d]
Hi-Line	1.0	1.0	1.0	0.0	1.0	0.0	0.0
161	1.0	1.0	1.0	0.0	1.0	0.0	0.0
HGB-19	1.5	4.0	1.0	4.0	4.5	1.0	4.0
HGB-12	1.0	4.5	1.0	4.0	4.0	1.5	6.0
HGAB-2	5.0	3.0	7.0	4.0	9.5	4.0	5.0
HGAB-3	6.0	4.0	8.0	5.0	12.0	6.0	8.0
HGA-3	5.0	1.0	8.0	0.0	8.0	2.0	0.0
HGA-1	3.0	1.0	4.5	0.0	3.5	2.0	0.0

a- Total *pina* and *pinb* expression was quantified using a 0.5X-6X scale, where 1 µg = 1X. Number is the average of two experiments

b- Total 'soft type' PINA and 'soft type' PINB was quantified using a scale that ranged from 0.5 X to 8X. Number is an average of two experiments

c- Sum of 'soft type' PINA and 'soft type' PINB

d- Bound PINA and PINB was quantified using a scale that ranged from 0.5Xto 8X. Number is an average of two experiments

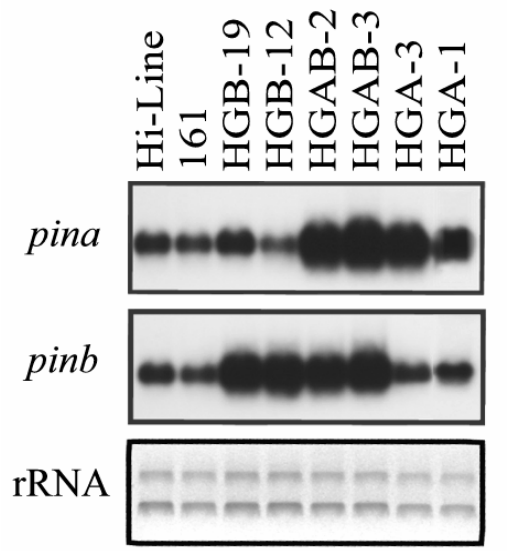

Figure 2. Total RNA was extracted from immature kernels at 21 days post anthesis. Each lane was loaded with 1 µg of total RNA and replicate blots were probed with P^{32} labeled 'soft type' *pina* and *pinb* sequences. rRNA was used as a loading control and shows no sign of variation on the duplicate ethidium bromide-stained agarose gel. In appropriate transgenic lines *pina* and *pinb* transcripts accumulated at much higher levels compared to the two control lines, Hi-Line and 161.

Total RNA was also extracted from developing wheat kernels at 7, 14, 21, and 28 DPA to determine a developmental expression pattern for the native and transgenic puroindolines (Figure 3). Two untransformed lines, Hi-Line and Heron, were compared to the transformed line HGB-12, which carries the 'soft type' *pinb-D1a* transgene. For Hi-Line, Heron, and HGB-12 there is no detectable transcripts of *pina* at seven DPA. Hi-Line, Heron, and HGB-12 show the highest transcript levels of *pina* between 14 and 28 DPA. The *pina* transcript levels observed in all three lines represent the accumulation of the native *pina-D1a* transcripts.

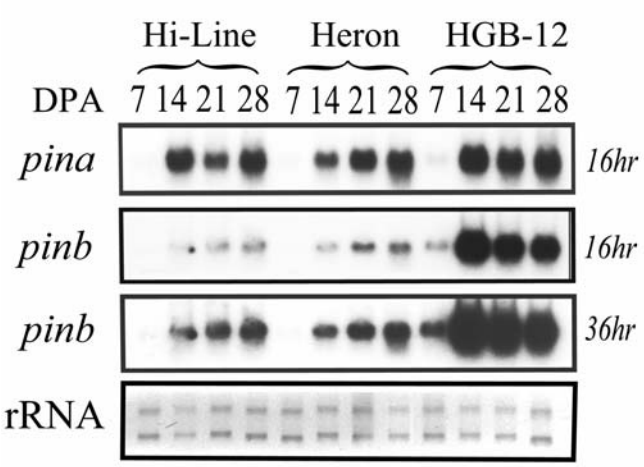

Figure 3. Total RNA was extracted from immature kernels at 7, 14, 21, and 28 days post anthesis (DPA). Each lane was loaded with 1 µg of total RNA and replicate blots were probed with P^{32} labeled 'soft type' *pina* and *pinb* sequences. rRNA was used as a loading control and shows no sign of variation, or RNA degradation, on the duplicate ethidium bromide-stained agarose gel. Hi-Line and Heron are untransformed varieties and the transgenic line HGB-12 expresses the 'soft type' *pinb* transgene. Numbers to the right of the figure indicate film exposure times.

In both of the untransformed cultivars there are no *pinb* transcripts seen at seven DPA, but in HGB-12 there is some accumulation of the 'soft type' *pinb* transcripts. In HGB-12 total *pinb* transcript levels are highest at 14 DPA, which then progressively

diminishes over time. In both Hi-Line and Heron there is a 'ramping-up' trend observed, with native *pinb* transcript levels steadily increasing over the 28 days. The accumulation of total *pinb* transcripts in HGB-12 at all collection points is around four times greater than what is observed for either of the untransformed varieties.

Total PINA and PINB Abundance

Triton X-114 protein extracts for selected lines were fractionated using SDS-PAGE and then visualized using a Coomassie Blue stain (Figure 4). The two PIN proteins can be resolved using this method and appear as two bands around 15 kDa. The top band is total PINA in the sample and the bottom band is total PINB (Giroux and Morris, 1998). The bands for both PINA and PINB are very weak in the two control lines, Hi-Line and 161, with the PINA band being more prominent. The value assigned to PINA in both these lines was 1.0 while the value of 'soft type' PINB in these lines is 0.0. 'Soft type' PINB is quantified as 0.0 in these lines because the PINB observed is coded for by the variant *pinb*-D1*b* sequence, which yields a non-functional or partially-functional puroindoline B protein. For this reason a unit of 1.0 was subtracted from all the transgenic lines PINB values, giving a value that represents the amount of 'soft type' PINB in those lines. HGB-19 shows an increase in 'soft type' PINB with a value of 4.0, while it retained a PINA value equal to 1.0. HGB-12 has a 'soft type' PINB level equal to 4.0 and a PINA level equal to 1.0. For HGAB-2 there was an increase in both PINA and 'soft type' PINB with levels equal to 7.0 and 4.0 respectively. HGAB-3 also showed an increase in puroindoline abundance with a PINA value of 8.0 and a 'soft type' PINB value of 5.0. For both HGA lines there was an increase in PINA abundance, with HGA-3

having a PINA value of 8.0 and HGA-1 having a PINA value of 4.5. Both of the HGA lines were given a 'soft type' PINB value of 0.0 (Table 2).

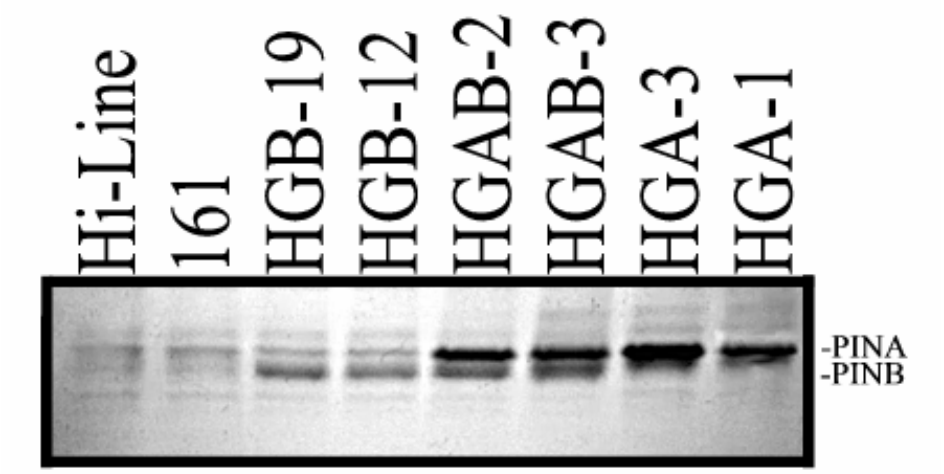

Figure 4. Using Triton X-114 detergent the puroindolines were extracted from 120 mg of coarsely milled whole-wheat flour. Proteins were fractionated via SDS-PAGE, and visualized using a Coomaisse Blue stain. The puroindolines can be resolved into two bands located around 15 kDa.

Friabilin is Associated With the Presence of Both PINA and PINB

PINA and PINB were extracted off the surface of starch granules, fractionated using SDS-PAGE, and then visualized using a Coomassie Blue stain (Figure 5). The puroindoline proteins that make up the protein marker friabilin can be resolved into two bands that are approximately 15 kDa in size. For the two control lines, Hi-Line and 161, there is no visible friabilin present giving them a value of 0 for both PINA and PINB. HGB-19 does show friabilin accumulation and its component proteins PINA and PINB have values of 1 and 4 respectively. HGB-12 also shows friabilin accumulation with a PINA value of 1.5 and a PINB value of 6. Friabilin is observed as well in HGAB-2 and HGAB-3, which have PINA values equal to 4 and 6 and PINB values equal to 5 and 8

respectively. HGA lines lack friabilin, with no band present for PINB and only a very faint band for PINA that's equal to 2 for both lines (Table 2). This faint band for PINA is not observed in either of the control lines.

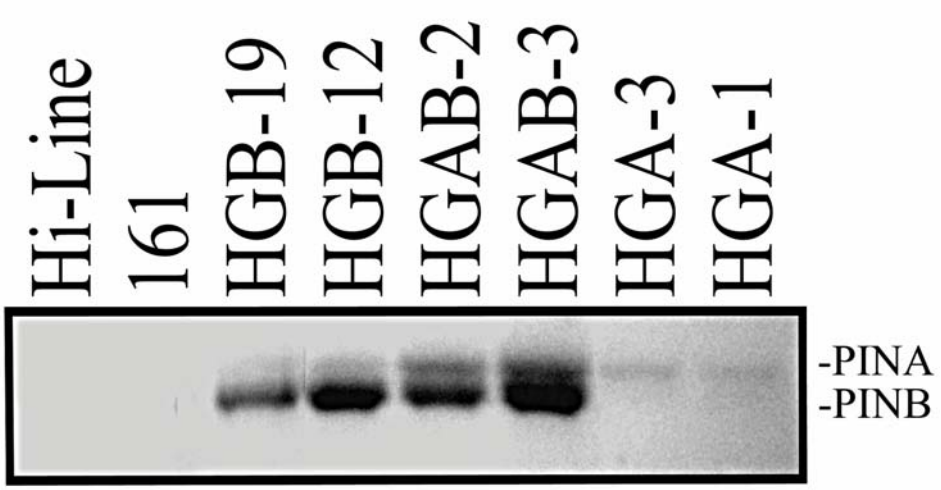

Figure 5. Friabilin was extracted off the surface of water-washed starch granules and fractionated using SDS-PAGE, then visualized using a Coomaisse Blue stain. The friabilin marker can be resolved into its component proteins, PINA and PINB, which are present around 15 kDa. In the two control lines and two HGA lines there is no friabilin present, whereas the HGB and HGAB lines have high levels of friabilin.

Friabilin was also extracted from Hi-Line, Heron, and HGB-12 kernel samples collected at 14 DPA, 21 DPA, 28 DPA, and maturity (Figure 6). For the hard wheat variety Hi-Line there is little to no friabilin present at any of the collection dates. In contrast the soft wheat variety Heron has friabilin present at all collection dates. Friabilin abundance in Heron steadily increased over time from 14 DPA to maturity. In the transformed HGB-12 line there is friabilin present at all collection times. Friabilin abundance in HGB-12 follows the same trend as seen in Heron, with a steady increase over time. When compared at each collection time the abundance of friabilin in HGB-12 samples is many times greater than that seen in the Heron samples.

Figure 6. Friabilin was extracted off the surface of water-washed starch granules isolated from Hi-Line, Heron, and HGB-12. Samples were collected over a developmental period of three weeks and at maturity. Friabilin extracts were fractionated using SDS-PAGE and visualized using a Coomaisse Blue stain. For the hard spring wheat Hi-Line there is no friabilin whereas in the soft spring wheat Heron and the transformed line HGB-12 friabilin increases during maturation.

Puroindoline Transformants Have Decreased Grain Hardness and Particle Size

Grain hardness was determined using the Single Kernel Characterization System (SKCS) and particle size index (Table 3). All transgenic lines were significantly softer than the two control lines Hi-Line and 161, which have SKCS values of 67.5 and 64.3 respectively. The greatest decrease in grain hardness was observed in HGB-12 which has an SKCS value of 24.2, followed by HGB-19 which has an SKCS value of 30.3. HGAB-2 was the next softest with an SKCS value of 31.8, followed by HGAB-3 which has a SKCS value of 34.0. HGA lines also have reduced grain hardness with HGA-3 having an SKCS value of 40.7 and HGA-1 having a slightly higher SKCS value of 44.3. Using a 90 μm sieve differences in flour particle size were observed between the transformants and controls. The HGB lines (HGB-19=18.4, HGB-12=19.8) and HGAB-2 (19.2) have almost a two-fold increase in the percent of flour recovered when compared to the

untransformed control Hi-Line (10.5). HGAB-3 (16.7) has a 59% increase in flour recovered, while HGA-3 (14.0) and HGA-1 (12.7) have only a 33.3% and 21% increase compared to Hi-Line respectively.

Table 3. Grain hardness determined by SKCS and particle size index for selected lines.

Line[a]	SKCS[b]	PSI%[c]
Hi-Line	67.5±3.8 a	10.5
161	64.3±1.0 a	13.1
HGB-19	30.3±4.9 c	18.4
HGB-12	24.2±3.1 d	19.8
HGAB-2	31.8±4.1 c	19.2
HGAB-3	34.0±3.9 c	16.7
HGA-3	40.7±4.6 b	14.0
HGA-1	44.3±6.8 b	12.7

a- All T_3 lines were derived from the spring wheat variety Hi-Line, which carries a 'soft type' *pina-D1a* sequence and a mutated *pinb-D1b* sequence. Homozygosity was confirmed using PCR, herbicide test, and amount of PIN in individual kernels.
b- Single kernel hardness values are an average of four replications. Means followed by the same letter do not differ from each other based on t-test at the 0.05 probability
c- Particle size index was determined using a 90 μm screen and 10 g of coarsely milled whole wheat flour.

Grain hardness was determined in the developmental study using only SKCS (Table 4). For Hi-Line, Heron, and HGB-12 grain hardness slowly decreased overtime from 21 DPA until maturity, which correlates with native and transgenic puroindoline expression (Figure 3) as well as friabilin accumulation (Figure 6). At maturity HGB-12 kernels were the softest with a SKCS value of 26.5, followed by Heron (43.2) and then Hi-Line (80.7). For HGB-12 at 14 DPA the kernels were the hardest with a SKCS value of 74.3, which concurs with the trend seen after 21 DPA in all lines. However for Hi-Line the 14 DPA kernels were the softest with an SKCS value of 69.6, and for Heron the 14 DPA kernels were of medium hardness with an SKCS value of 52.0.

Table 4. Developmental hardness analysis of selected lines using SKCS.

Line[a]	DPA[b]	SKCS[c]	Stan. Dev.[d]
Hi-Line	14	69.6	30.4
Hi-Line	21	105.1	16.0
Hi-Line	28	90.5	15.0
Hi-Line	Mature	80.7	15.1
Heron	14	52.0	22.0
Heron	21	74.7	13.9
Heron	28	48.4	12.8
Heron	Mature	43.2	13.1
HGB-12	14	74.3	19.3
HGB-12	21	49.6	12.9
HGB-12	28	27.6	12.2
HGB-12	Mature	26.5	14.2

a- HGB-12 was derived from the spring wheat variety Hi-Line
b- Number of days post anthesis (DPA) when seeds were collected.
c- Single kernel hardness values based on one replication using >80 seeds.
d- Standard deviation between individual kernels in the one replication.

Discussion

Our objective was to identify and characterize the puroindolines role in controlling grain hardness. To do so the hard spring wheat variety Hi-Line, which carries the *pina-D1a* and *pinb-D1b* sequences, was transformed with 'soft type' puroindoline a (*pina-D1a)*, puroindoline b (*pinb-D1a*), or both. Truly isogenic lines were obtained which allowed us to observe the effects these two puroindoline genes have on grain hardness independently and together. The puroindolines make up the protein marker friabilin (Jolly et al. 1993; Morris et al. 1994) which can be used to distinguish between hard and soft wheats (Greenwell and Schofield, 1986). Consequently it has been suggested that the puroindolines are the causal genes for grain hardness in wheat (Giroux and Morris 1998).

In support of this theory Campbell et al. (1999) reported that the segregation of the *Ha* locus, which contains the closely linked genes *pina* and *pinb* (Sourdille et al. 1996; Giroux and Morris 1997; Turnbull et al. 2003), in a hard X soft wheat cross accounted for approximately 60% of the variation seen in wheat grain hardness. It was later shown in transgenic rice the addition of the puroindolines resulted in softer rice kernels that had a lower particle size index and less starch damage (Krishnamurthy et al. 2001). Rice was a model system to study the puroindolines because, like sorghum and maize, it contains no puroindoline homologs (Gautier et al. 2000). Further supporting evidence was put forth when Beecher et al. (2002) reported that the complementation of the *pinb-D1b* mutation with the 'soft-type' *pinb-D1a* sequence in hard spring wheat resulted in grain hardness, particle size, and starch granules characteristic of soft wheats.

The results of our experiments concur with the findings described above and support the hypothesis that the puroindolines are the primary genetic elements in determining grain hardness. In lines transformed with both *pina-D1a* and *pinb-D1a* sequences, and just the *pinb-D1a* sequence, there was increased puroindoline transcript levels (Figure 2; Table 2), increased puroindoline content (Figure 4; Table 2), and a phenotype typical of soft wheats. This phenotype was characterized by the presence of the protein marker friabilin (Figure 5), decreased grain hardness (Table 3), and decreased flour particle size (Table 3). In lines transformed exclusively with the *pina-D1a* sequence there was an increase in *pina* transcript levels (Figure 2; Table 2) and PINA content (Figure 4; Table 2), with a slight reduction in grain hardness and flour particle size (Table 3). The differences observed between the two HGA lines are likely due to the

fact that HGA-1 is a heterozygote (Table 1). This would account for the decreased transcript levels of *pina* and decreased PINA accumulation seen in HGA-1 compared to HGA-3. The reductions in grain hardness and flour particle size were significant in HGA lines but were not as dramatic as those seen in the HGB and HGAB lines, which correlates with the absence of friabilin (Figure 5). Based on these results there is evidence that decreased levels of grain hardness are associated with the presence of both 'soft type' puroindoline proteins and not total puroindoline content. This is consistent with the fact that all wheats characterized to date have a mutation in one of their puroindoline genes, while soft wheats possess the 'soft type' *pina-D1a* and *pinb-D1a* sequences (Giroux and Morris 1998; Lillemo and Morris 2000; Morris et al. 2001). We can also report that grain softness in developing wheat kernels is correlated with both puroindoline transcript levels (Figure 3; Table 4) and friabilin accumulation (Figure 6; Table 4). A limitation of this study is that the variety Hi-Line contains the altered *pinb-D1b* allele and a 'soft type' *pina-D1a* allele. Therefore, it is difficult to determine if the reduction in grain hardness in the HGA lines is limited due to native expression of 'soft type' PINA-D1a. If so, this would indicate that there is a limit to the amount of PINA and PINB that can bind to starch granules. However, this data still indicates that a soft phenotype results from the presence of both PINA-D1a and PINB-D1a.

These unique isogenic lines will furthermore be useful in providing insight into the effects of puroindoline content and grain hardness on a broad range of milling and baking qualities. The reconstitution of puroindolines in flour has been shown to have effects on dough foaming properties, crumb grain, and loaf volume (Dubreil et al. 1998).

Recently Martin et al. (2001) demonstrated that variation in puroindoline sequence within a recombinant inbred population was associated with several milling and baking traits such as milling and break flour yield, milling score, crumb grain score, loaf volume, and flour ash.

Besides their involvement in grain hardness and several milling and baking traits, the puroindolines also have anti-fungal properties. In transgenic rice that constitutively expressed the puroindolines there was an increase in disease tolerance to the two foliar pathogens *Magnaporthe grisea* and *Rhizoctonia solani* (Krishnamurthy et al. 2001). The experimental material characterized here could also be useful for studying the puroindolines anti-fungal properties *in-vivo* by assessing their effectiveness against seed borne pathogens such as *Penicillium* spp.

In this study it has been shown that grain hardness in hexaploid wheat is not correlated with total puroindoline content but instead with the presence of both functional PINA and PINB suggesting some type of synergism. These conclusions are paralleled by the fact that grain hardness in developing wheat kernels is correlated with both puroindoline transcript levels and friabilin accumulation. These results provide further evidence to support the hypothesis that the puroindolines are the causal genes for wheat grain hardness.

CHAPTER 3

HARD WHEAT MILLING AND BREAD BAKING TRAITS AFFECTED BY THE SEED-SPECIFIC OVER-EXPRESSION OF PUROINDOLINES.

Introduction

For commercial purposes wheat grain is classified as hard or soft based on endosperm texture. Differences in endosperm texture have far reaching effects on numerous milling and end use qualities in wheat. Soft textured wheats typically have decreased flour yields, increased break flour yields, smaller flour particle size, and less starch damage compared to hard textured wheats (Symes 1965; Symes 1969; Pomeranz et al 1984). For baking purposes soft textured wheats are generally used to make cookies and pastries, while hard textured wheats are typically used to make breads (Morris and Rose, 1996). The Hardness (*Ha*) locus located on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978) differentiates between soft and hard grain texture (Symes 1965; Baker 1977).

The discovery of the protein friabilin on the surface of water-washed starch granules provided a biochemical way to distinguish between hard and soft wheats (Greenwell & Schofield 1986). The presence of the protein friabilin on water-washed starch is associated with soft endosperm texture, whereas water-washed starch from hard textured wheats is essentially devoid of friabilin. Further investigation into the composition of friabilin revealed that the 15 kDa protein was comprised primarily of two

basic cysteine-rich proteins, puroindoline-A (PINA) and puroindoline-B (PINB) (Jolly et al. 1993; Morris et al. 1994), and that friabilin was under the genetic control of chromosome 5D (Jolly et al. 1993). PINA and PINB also have a unique tryptophan domain (Gautier et al 1994) that is hypothesized to be an active site for the binding of phospholipids found on the surface of starch granules (Gautier et al. 1994; Marion et al. 1994).

The tightly linked genes encoding for puroindoline-a (*pina-D1a*) and puroindoline-b (*pinb-D1a*) have since been cloned and identified as residing at the *Ha* locus (Gautier et al. 1994; Sourdille et al. 1996; Giroux and Morris 1997, 1998; Tranquilli et al. 1999), providing a genetic basis for the control of grain texture in wheat. Recent surveys of wheat genotypes across the world has revealed that soft wheats all have the same *pin* alleles (*pina-D1a*; *pinb-D1a*), while all hard wheats have a mutation in either *pina* or *pinb (*Giroux & Morris 1998; Lillemo & Morris 2000; Morris et al 2001). To date there are seven *pin* mutations characterized (reviewed in Morris 2002), with the most prevalent being a null mutation in *pina* (*pina-D1b*) and a point mutation in *pinb* (*pinb-D1b*) that results in a glycine-to-serine switch at the 46^{th} residue of the peptide (Giroux & Morris 1997, 1998). To support the hypothesis that the puroindolines are the primary genetic elements controlling grain texture in wheat the use of plant transformation has been employed in both rice (Krishnamurthy & Giroux 2001) and wheat (Beecher et al. 2002; Hogg et al. 2004). The transgenic addition of the puroindolines to both rice and a hard spring wheat cultivar, possessing the *pinb-D1b* allele, resulted in significantly softer endosperm texture, reduced endosperm particle size,

and reduced starch damage. A positive association was also found between puroindoline transcript levels, friabilin accumulation, and soft grain texture in developing transgenic wheat kernels (Hogg et al. 2004).

Grain texture has been shown to affect many milling and bread baking characteristics in hard wheat (Slaughter et al. 1992; Ohm et al 1998) as has puroindoline sequence type. *Pinb* was shown to be a major QTL for flour yield, grain texture, starch damage, alkaline-water retention capacity (AWRC), and dough water absorption in a recombinant inbred population segregating for *pinb-D1a* (soft) and *pinb-D1b* (hard) alleles (Campbell et al. 2001). In a hard wheat recombinant inbred population segregating for the two most common *pin* mutations it was found that lines possessing the *pinb-D1b* mutation had higher break flour yields, higher flour yields, lower flour ash, improved crumb grain scores, and larger loaf volumes compared to lines carrying the *pina-D1b* mutation (Martin et al. 2001). Cane et al. (2004) supports these findings by reporting that in select Australian breeding cultivars, hard cultivars possessing the *pinb-D1b* mutation had higher flour yields, lower water absorption, and a smaller particle size than those hard cultivars possessing the *pina-D1a* mutation. The reconstitution of wheat flour with 0.1% PINA was also reported to have significant affects on both crumb grain scores and bread loaf volumes (Dubreil et al. 1998).

Our objectives here were to characterize milling and baking traits affected by the over-expression of puroindolines *in vivo* and to evaluate milling performance of selected lines at a semi-commercial scale. To do so we have transgenically created a set of

isogenic lines in a hard spring wheat background that over-express *pina-D1a, pinb-D1a,* or both *pina-D1a* and *pinb-D1a* solely in the seed endosperm.

Materials and Methods

Transgenic lines used in this study were derived as described by Beecher et al. (2002) and Hogg et al. (2004). To achieve over-expression the puroindoline genes (*pina-D1a* and *pinb-D1b*) were placed between the glutenin gene flanking regulatory sequences from pGlu10H5 (Blechl and Anderson 1996). A total of 17 different homozygous transgenic lines were created which had added *pina-D1a*, *pinb-D1a*, or both *pina-D1a* and *pinb-D1a*. All lines were co-transformed with the *Bar* expression vector pRQ101A (Sivamani et al. 2000), which confers resistance to the herbicide glufosinate (AgrEvo USA Company, Willmington, DE). The cultivar 'Hi-Line' (Lanning et al. 1992) was used for transformation, which carries the wild-type *pina-D1a* sequence and the variant *pinb-D1b* sequence (Giroux et al. 2000). Hi-Line did not differ from 161, Hi-Line transformed with *Bar* only, for any of the measured traits. Thus, these two lines without added *pin* were collectively considered the hard wheat control group.

To re-confirm genotype identity DNA was extracted (Riede et al. 1996) from homozygous T_3 leaf samples representing each experimental line. Genomic DNA was digested with *HindIII*. Southern blot analysis was performed (Beecher et al. 2002) and replicate blots were probed with P^{32} labeled *pina-D1a* and *pinb-D1a* sequences. Total PINA and PINB proteins in homozygous T_3 seeds were visualized for all experimental lines following methods described by Giroux et al. (2003). To quantify puroindoline

protein expression a scale ranging from 1 to 8, in multiples of 1, was used. This scale was constructed using a Hi-Line TX-114 protein extract where 1X equaled 5 µL of sample (120 µL buffer/100 mg seed powder). As controls, seeds from the durum (*Triticum Turgidum* L.) cultivar 'Langdon' and the soft substitution line Langdon-5D (5B) were also analyzed for puroindoline presence and quantity.

Eighteen T_3 experimental lines plus Hi-Line were grown during the summer of 2003 at the Montana State University Arthur H. Post Farm using a randomized block design with two replications. Plots were four 3 m rows with 30 cm between rows. The experiment was grown in irrigated and non-irrigated environments. At maturity grain from all four rows from each plot was harvested with a plot combine, and 200 T_4 seeds from each plot were used for grain texture (SKCS hardness index), kernel diameter, and kernel weight measurements using the Single Kernel Characterization System 4100 (Perten Instruments North America Inc., Springfield, IL). Particle size index (%) (PSI) was also measured using a sub-sample of grain from each plot following Method 55-30 (AACC, 2000) with the following change; a 90 µm sieve was used in place of a 70 µm sieve. Grain protein content was determined for each plot by near-infrared transmission (NIT) using an Infratec 1225 Grain Analyzer (Foss North America Inc., Eden Prairie, MN).

Following kernel analysis 500 g of seed from each plot was tempered to 13.0 % moisture (fresh-weight basis) for 15 h. Samples were then milled using Quadrumat mills following the modifications of Jeffers and Rubenthaler (1977). "Straight-grade" flour was analyzed for moisture (Method 44-16), protein (Method 46-30), and ash (Method 08-

01) (AACC, 2000). All flour parameters are reported at a 14% moisture basis. Mixograph analysis was performed using a 10-g instrument following method 54-40A (AACC, 2000). Bread was then baked following Method 10-10B (AACC, 2000). Loaf volume was determined using a canola seed displacement method and crumb grain score was rated from 0 (unsatisfactory) to 5 (excellent).

From each replication of Hi-Line, HGA-13, HGB-6, and HGAB-18 from the irrigated environment 300 g of seed was milled into whole wheat flour using a UDY Cyclone mill with a 0.5 mm screen (UDY Co., Fort Collins, CO). Whole wheat flour was analyzed for protein content, ash content, and mixograph properties following the procedures described. Whole wheat flour was then used to make bread for which loaf volume and crumb grain score were determined following described procedures with the following exception; 300 ppm absorbic acid was used in place of 150 ppm absorbic acid.

Data were analyzed via analysis of variance combined over environments. Environments and entries were considered fixed effects and replications as random effects using PROC GLM in SAS (SAS Institute, 1988). Least significant difference (LSD) was computed to compare individual mean differences, and contrasts of linear combinations of means were used to compare group means. Correlations among traits were computed using entry means.

Hi-Line and HGAB-18 were chosen to investigate differences in milling characteristics and “straight-grade” flour production on a semi-commercial scale using a Miag Multomat pilot-scale mill (Langerringen, Germany). The Miag mill produces 14 mill streams per sample, and was operated at 2 bu (about 55 kg) per hour. There are three

break passes and five reduction passes on this mill that generate five feed streams and nine flour streams. The nine flour streams were blended to produce “straight-grade” flour.

The cultivar Hi-Line was tempered to 16% moisture and HGAB-18 was tempered to 13% moisture 18 h prior to milling. Generally, soft-textured wheat is tempered to 14.5% moisture for this milling system, but the temper level was reduced due to the very soft endosperm texture of HGAB-18. Break rolls were adjusted so that 43% of the first break release products passed through a #24 Tyler (707 µm) wire screen in 20 s of sifting. The second break was adjusted so that 64% of the release products were produced using the same sifting treatment. The third break roll was adjusted to clean the bran as completely as possible without excessive shattering of the bran. Reduction rolls were adjusted by observation of the stock to produce as much flour as possible without over-grinding and flaking the stock.

The 14 mill streams and the “straight grade” flour were analyzed for ash content (AACC method 08-01), moisture (AACC method 44-16) and protein content (AACC method 46-30; N X 5.7). Stream weights and their respective ash and protein contents were adjusted to a constant 14% moisture basis. To produce cumulative ash and protein curves, each stream was ordered by increasing ash or protein content. Mill stream content was plotted, beginning with the stream with the lowest content, against the percentage of total products it represented. Sequentially, the next lowest stream was mathematically “added” to the previous stream(s), the total cumulative content(s) and percentage of total products then recalculated and plotted.

Results and Discussion

To determine milling and baking traits associated with the over-expression of puroindolines *in vivo* a set of 18 transgenic lines was created in a hard red spring wheat background (Beecher et al. 2002; Hogg et al. 2004). The presence and expression of the puroindoline transgenes in these lines was re-confirmed in the T_3 generation using Southern blot analysis (Table 5) and Triton X-114 protein extracts (Figure 7; Table 5). Based on Southern analysis there were four transformed lines identified as possessing the *pina-D1a* transgene (HGA: 1, 3, 5, 13), eight lines positive for the *pinb-D1a* transgene (HGB: 2, 5, 6, 12, 16, 19, 21, 35), and five lines possessing both *pina-D1a* and *pinb-D1a* transgenes (HGAB: 1, 2, 3, 11, 12, 18). The transgenic line 161 and untransformed Hi-Line were both negative for the presence of non-native puroindoline genes (Table 5). Northern blot analysis was performed previously on eight of these lines (Hi-Line, 161, HGA-1, HGA-3, HGB-12, HGB-19, HGAB-2, and HGAB-3). In lines transformed with the puroindolines there was approximately a four to five-fold increase in the appropriate puroindoline transcript levels compared to the controls Hi-Line and 161 (Hogg et al. 2004).

Table 5. Southern blot results and puroindoline protein expression levels for controls and lines transformed with *pina-D1a, pinb-D1a,* or both *pina-D1a* and *pinb-D1a.*

	Transgenes[b]				
ID[a]	*pina*	*pinb*	PINA[c]	PINB[c]	Total PIN
Hi-Line	-	-	1.0	1.0	2.0
161	-	-	1.0	1.0	2.0
HWC[d]	-	-	**1.0**	**1.0**	**2.0**
HGA-1	+	-	7.0	1.0	8.0
HGA-3	+	-	8.0	1.0	9.0
HGA-5	+	-	8.0	1.0	9.0
HGA-13	+	-	7.5	1.0	8.5
HGA AVG	+	-	**7.6**	**1.0**	**8.6**
HGB-2	-	+	1.0	2.0	3.0
HGB-5	-	+	1.0	1.5	2.5
HGB-6	-	+	1.0	3.5	4.5
HGB-12	-	+	1.3	3.8	5.0
HGB-16	-	+	1.0	2.0	3.0
HGB-19	-	+	1.0	3.5	4.5
HGB-21	-	+	1.0	2.0	3.0
HGB-35	-	+	1.0	1.8	2.8
HGB AVG	-	+	**1.0**	**2.4**	**3.4**
HGAB-2	+	+	7.0	3.5	10.5
HGAB-3	+	+	8.0	5.0	13.0
HGAB-11	+	+	8.0	3.3	11.3
HGAB-12	+	+	5.0	1.5	6.5
HGAB-18	+	+	6.0	4.5	10.5
HGAB AVG	+	+	**6.8**	**3.6**	**10.4**

a- All homozygous T_3 lines were derived from the hard spring wheat Hi-Line, which carries the *pina-D1a* and *pinb-D1b* alleles

b- Southern blot analysis was performed on T_3 genomic DNA extracts, which were digested with *Hind*III and hybridized with *pina-D1a* and *pinb-D1a* PCR generated probes. Lines denoted as + were PCR and Southern blot positive for the *pina-D1a* and *pinb-D1a* transgenes

c- Puroindoline A (PINA) and puroindoline B (PINB) proteins were quantified using a scale that ranged from 1 to 8 on an acrylamide gel. Number shown is an average of two experiments

d- HWC denotes average of hard wheat controls (Hi-Line and 161)

To establish protein expression levels for all experimental lines the puroindoline proteins, PINA and PINB, were extracted from mature seeds using a Triton X-114 method (Giroux et al. 2003) and visualized via non-denaturing SDS-PAGE and Coomaisse blue staining. The durum cultivar Langdon and the substitution line Langdon-5D (5B) were used as negative and positive controls respectively. As in all durum wheats, Langdon lacks the D-genome and consequentially the *Ha* locus that is found on 5DS (Mattern et al. 1973; Law et al. 1978). Langdon is therefore devoid of both PINA and PINB (Figure 7). However, in the substitution line Langdon-5D (5B) both PINA and PINB are present (Figure 7) (Giroux and Morris 1997, 1998). The HGA lines exhibited an increase in PINA that was on average 7.6 times greater than the amount of PINA in Hi-Line and 161 (Figure 7; Table 5). The HGB lines showed increased amounts of PINB that were on average 3.4 times greater than those seen in Hi-Line and 161 (Figure 7). The HGAB lines had increased levels of both PINA and PINB and possessed the highest total puroindoline protein content out of all the lines examined with average values of 6.8 and 3.6 for PINA and PINB respectively (Figure 7). Hi-Line and 161 had similar puroindoline protein expression levels and profiles, with PINA being more prominent than PINB (Figure 7). The PINB band seen for Hi-Line and 161 represents the mutant peptide that is coded for by the *pinb-D1b* allele. This peptide is considered to be non-or partially functional due to a glycine-to-serine change at the 46th residue near the proteins unique tryptophan domain (Giroux and Morris 1998). It is hypothesized that this tryptophan domain is an active site for the binding of phospholipids found on the surface of the starch granules (Gauiter et al. 1994; Marion et

al. 1994). Based on this, the true value of functional PINB in all lines might be obtained by subtracting a value of 1 from their given PINB values (Table 5).

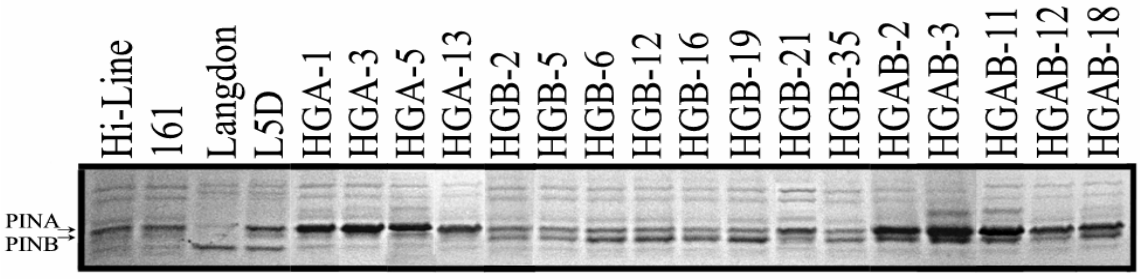

Figure 7. Puroindoline proteins, PINA and PINB, were fractionated via SDS-PAGE and can be resolved into two bands around 15 kDa. The negative durum control Langdon has neither PINA nor PINB, whereas both are present in the positive control Langdon-5D (5B) denoted as L5D.

A very strong negative correlation ($p<0.01$; $r = -0.94$) was found between SKCS hardness index and PSI, which corroborates reports by others (Pomeranz et al. 1985; Morris and Massa 2003). The hard wheat control (HWC) group had a SKCS hardness mean of 83.6 and a particle size index (PSI) mean of 8.54% (Table 6). As a group the HGA lines had a SKCS hardness mean of 51.9 and a PSI mean of 13.4% (Table 6), while the HGB lines had a much softer endosperm texture with a SKCS hardness mean of 29.0 and a PSI mean of 15.4% (Table 6). The HGAB lines had the softest endosperm texture with SKCS hardness and PSI means of 23.2 and 17.9%, respectively (Table 6). The HWC group, the HGA group, the HGB group, and the HGAB group were all significantly different from each other for both SKCS hardness and PSI values (Table 6). Using a relatively small number of transgenic *pin* addition lines, Hogg et al. (2004) reported that total puroindoline content did not correlate well with changes in grain hardness unless both functional PINA and PINB were present. The results here with a

much large data set agree with those findings. For example, even though the HGA lines have high levels of total puroindoline (Table 5), they are intermediate in grain hardness (Table 6) since they lack functional PINB and cannot form high levels of starch-surface friabilin (Hogg et al. 2004). However, when high levels of both functional PINA and PINB are present the softest textured is achieved, exemplified by the HGAB lines (Tables 5 and 6).

The mean grain protein content for the HGB lines (176 $g\text{-}kg^{-1}$) and HGAB lines (177 $g\text{-}kg^{-1}$) was significantly higher compared to the HWC group (171 $g\text{-}kg^{-1}$), while the mean grain protein for the HGA lines (172 $g\text{-}kg^{-1}$) was not significantly different from the HWC group (Table 6). The increased protein content observed in the HGB and HGAB lines is likely a result of their decreased kernel diameter and weight (Table 6). There are significant negative correlations between wheat protein and kernel diameter ($p<0.01$; $r = -0.75$), and between wheat protein and kernel weight ($p<0.05$; $r = -0.45$). The relationship between kernel size and protein content observed here is in agreement with the findings of others (Ohm et al 1998). The transgenic lines as groups also had significantly lower flour protein than the HWC group (Table 6). This is not unexpected since the outer endosperm layers, which contain most of the protein, are not as easily separated from the bran and aleurone layers during the milling process as they are in hard wheats. Accordingly, the HGA group, which is intermediate in hardness, possesses more flour protein compared to the extremely soft textured HGB and HGAB groups (Table 6).

Table 6. Kernel characteristics, measurements of grain texture, and protein content based on mean of two environments for controls and transformed lines.

ID[a]	Grain protein	Flour protein	Grain hardness		Kernel weight	Kernel diameter
			SKCS[b]	PSI[c]		
	g kg^{-1}				mg	mm
Hi-Line	170	145	85.0	8.19	26.8	2.22
161	172	145	82.2	8.89	28.5	2.33
HWC[d]	**171**	**145**	**83.6**	**8.54**	**27.7**	**2.27**
HGA-1	169	136	56.6	12.7	28.3	2.24
HGA-3	170	133	48.5	14.0	27.1	2.13
HGA-5	180	139	48.8	13.3	26.0	2.10
HGA-13	171	137	53.6	13.5	28.1	2.19
HGA AVG	**172**	**136**	**51.9**	**13.4**	**27.4**	**2.17**
HGB-2	182	133	37.1	13.4	26.4	2.07
HGB-5	180	127	28.2	16.2	25.5	2.02
HGB-6	172	124	23.1	15.8	26.9	2.11
HGB-12	172	119	21.7	15.9	26.6	2.07
HGB-16	179	129	35.7	14.8	23.7	1.97
HGB-19	175	123	24.2	16.8	25.4	1.99
HGB-21	168	122	35.1	14.1	27.8	2.08
HGB-35	179	127	26.6	16.0	24.6	1.99
HGB AVG	**176**	**126**	**29.0**	**15.4**	**25.9**	**2.04**
HGAB-2	197	147	28.0	17.8	23.8	1.99
HGAB-3	161	112	27.6	15.8	28.2	2.07
HGAB-11	169	115	21.1	18.3	24.9	1.95
HGAB-12	182	133	24.5	18.9	25.0	2.00
HGAB-18	175	124	14.9	18.7	25.7	2.00
HGAB AVG	**177**	**126**	**23.2**	**17.9**	**25.5**	**2.00**
LSD (0.05)[e]	5.0	2.9	2.00	0.79	1.03	0.06
LSD (0.01)[e]	7.1	4.1	2.87	1.13	1.48	0.09
HWC vs. HGA[f]	0.4885	<.0001	<.0001	<.0001	0.4863	<.0001
HWC vs. HGB[f]	0.0059	<.0001	<.0001	<.0001	<.0001	<.0001
HWC vs. HGAB[f]	0.0022	<.0001	<.0001	<.0001	<.0001	<.0001
HGA vs. HGB[f]	0.0192	<.0001	<.0001	<.0001	<.0001	<.0001
HGA vs. HGAB[f]	0.0067	<.0001	<.0001	<.0001	<.0001	<.0001
HGB vs. HGAB[f]	0.4464	0.2454	<.0001	<.0001	0.2102	0.0241

a- All homozygous T_3 lines were derived from the hard spring wheat Hi-Line, which carries the *pina-D1a* and *pinb-D1b* alleles

b- Single kernel characterization system hardness index values

c- Particle size index (%)

d- HWC denotes average of hard wheat controls (Hi-Line and 161)

e- LSD denotes least significant differences and values are used to compare individual transgenic lines vs. Hi-Line

f- For group comparisons p< values are given

All lines were tempered to 13% moisture prior to Quadrumat milling. This level of moisture is optimum for soft wheats but below optimum for hard wheats. The over-expression of the puroindolines in all transgenic groups resulted in increased break flour yields, lower flour yields, and lower ash values compared to the HWC group (Table 7). The HGAB group however was the only group that significantly differed from the HWC group for both mixograph absorption and mix time (Table 7). It was surprising that neither the HGA group nor the HGB group were significantly different from the HWC group for these two characteristic given their severe reductions in grain hardness (Table 7). The transgenic addition of puroindolines to Hi-Line principally affected grain texture and milling characteristics, but also detrimentally affected some bread baking qualities. When compared as groups the HGA lines, HGB lines, and HGAB lines all had significantly lower loaf volumes compared to the HWC group (Table 7). The HGAB group also had a significantly less desirable crumb grain score than the HWC group (Table 7).

Table 7. Milling and baking traits based on mean of two environments for controls and lines transformed with *pina-D1a, pinb-D1a,* or both *pina-D1a* and *pinb-D1a.*

ID[a]	Flour Yield	Break flour Yield	Flour ash	Mixograph absorption	Mix time	Loaf volume	Crumb grain[b]
	g kg^{-1}				min	cc	
Hi-Line	616	688	4.93	676	5.03	1410	3.50
161	626	704	4.80	673	5.10	1377	3.50
HWC[c]	**621**	**696**	**4.87**	**674**	**5.07**	**1394**	**3.50**
HGA-1	551	789	3.70	649	4.90	1273	3.75
HGA-3	573	807	4.00	649	5.13	1254	3.50
HGA-5	527	829	4.20	635	3.53	1226	2.75
HGA-13	553	823	4.30	638	4.45	1284	3.75
HGA AVG	**551**	**812**	**4.05**	**643**	**4.50**	**1258**	**3.44**
HGB-2	511	770	4.20	643	3.68	1279	3.25
HGB-5	487	828	3.90	633	5.05	1284	3.75
HGB-6	504	805	3.80	631	4.27	1330	3.00

Table 7 continued

HGB-12	483	838	3.70	617	4.98	1096	3.25
HGB-16	446	786	4.00	631	6.48	1284	3.25
HGB-19	486	809	3.70	615	3.43	1121	3.50
HGB-21	510	804	3.80	614	4.20	1271	3.50
HGB-35	483	789	3.90	634	5.78	1270	3.25
HGB AVG	**488**	**804**	**3.88**	**627**	**4.73**	**1242**	**3.34**
HGAB-2	468	882	4.10	671	3.43	1346	1.50
HGAB-3	488	826	3.30	563	1.25	526	1.25
HGAB-11	480	853	3.50	570	2.48	726	2.00
HGAB-12	513	832	4.20	488	4.30	1322	2.67
HGAB-18	517	849	3.80	630	5.15	1153	3.00
HGAB AVG	**493**	**848**	**3.78**	**584**	**3.32**	**1015**	**2.08**
LSD(0.05)[d]	38.1	45.4	0.20	102	0.96	45.71	0.55
LSD(0.01)[d]	54.6	65.1	0.30	146	1.38	65.57	0.79
HWC vs. HGA[e]	<.0001	<.0001	<.0001	0.4175	0.1287	<.0001	0.7782
HWC vs. HGB[e]	<.0001	<.0001	<.0001	0.1766	0.3103	<.0001	0.4288
HWC vs. HGAB[e]	<.0001	<.0001	<.0001	0.0188	<.0001	<.0001	<.0001
HGA vs. HGB[e]	<.0001	0.0171	0.0127	0.6053	0.4114	0.2132	0.5819
HGA vs. HGAB[e]	<.0001	0.5344	<.0001	0.0813	0.0004	<.0001	<.0001
HGB vs. HGAB[e]	0.6412	0.0008	0.0246	0.1254	<.0001	<.0001	<.0001

a- All homozygous T_3 lines were derived from the hard spring wheat Hi-Line, which carries the *pina-D1a* and *pinb-D1b* alleles
b- Based on 0 (unsatisfactory) to 5 (excellent) scale
c- HWC denotes average of hard wheat controls (Hi-Line and 161)
d- LSD denotes least significant difference and values given are to compare individual transgenic lines vs. Hi-Line
e- For group comparisons p values are given

Single kernel hardness was positively correlated with kernel diameter and kernel weight, while PSI was negatively correlated with these same traits (Table 8). There was however no significant correlation between grain protein and either measurement of endosperm texture (Table 8), which concurs with the findings of others (Pomeranz et al. 1985; Ohm et al. 1998). Very strong positive correlations were found for SKCS hardness vs.: flour yield (r = 0.87) (Figure 8), flour ash (r = 0.79), and flour protein (r = 0.72) (Table 8). On the contrary a very strong negative correlation was found between SKCS hardness and break flour yield (r = -0.81) (Table 8; Figure 9). The inverse relationships

were found for PSI vs.: flour yield (r = -0.78), flour protein (r = -0.57), flour ash (r = -0.71), and break flour yield (r = 0.88) (Table 8). Flour yield, break flour yield, flour protein, and flour ash content were all highly interrelated (Table 9). These results reaffirm previous findings that variation in puroindoline sequence type and associated grain hardness differences influence many milling characteristics (Campbell et al 2001; Martin et al 2001; Cane et al. 2004).

Table 8. Correlations between grain hardness and kernel traits, milling traits, and bread baking traits for 17 transgenic and two control lines based on mean of two environments.

Variable	SKCS[a]	PSI%[b]
Kernel weight	0.46*	-0.55**
Kernel diameter	0.82**	-0.83**
Wheat protein	-0.17	0.32
Flour yield	0.82**	-0.77**
Break flour yield	-0.79**	0.87**
Flour ash	0.73**	-0.67**
Flour protein	0.72	-0.55**
Mixograph absorption	0.52**	-0.55**
Mix time	0.27	-0.25
Loaf volume	0.43*	-0.38*
Crumb grain score	0.39*	-0.44*

*, ** significantly different from zero at 0.05 and 0.01 probability levels, respectively
a- Single kernel characterization system
b- Particle size index

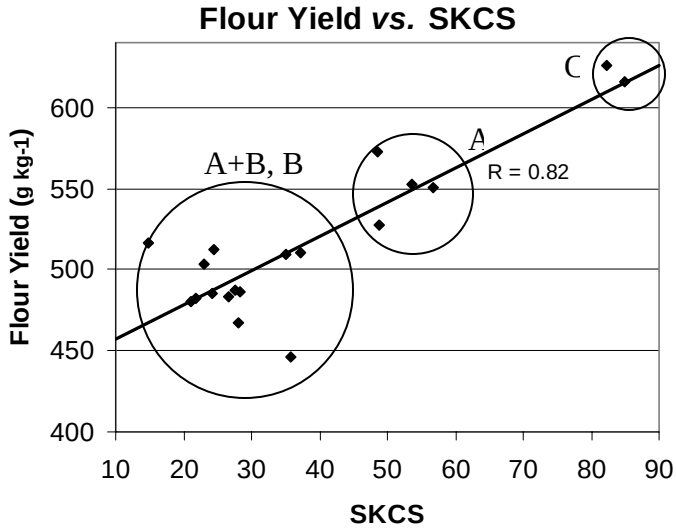

Figure 8. Plot showing positive correlation between flour yield and SKCS for all 19 lines. C=hard wheat controls, A = *pina-D1a* transformants, and A+B, B = *pina-D1a+pinb-D1b, pinb-D1b* transformants. Data points are based on means of two environments. SKCS denotes Single Kernel Characterization System hardness index values.

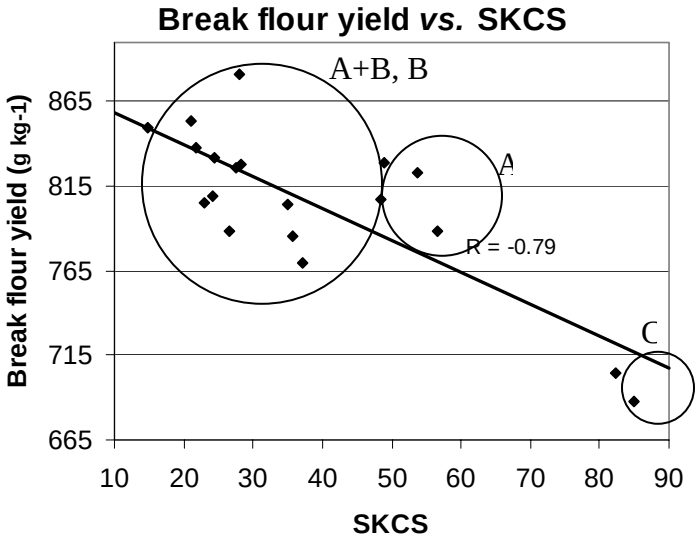

Figure 9. Plot showing negative correlation between break flour yield and SKCS for all 19 lines. C=hard wheat controls, A = *pina-D1a* transformants, and A+B, B = *pina-D1a+pinb-D1b, pinb-D1b* transformants. Data points are based on means of two environments. SKCS denotes Single Kernel Characterization System hardness index values.

Table 9. Correlations among milling and bread baking traits for 17 transgenic and two control lines based on mean of two environments.

Trait	Flour yield	Break flour yield	Flour ash	Flour protein	Loaf volume
Break flour yield	0.68**				
Flour ash	0.72**	-0.66**			
Flour protein	0.51**	-0.40*	0.75**		
Loaf volume	0.33	-0.39*	0.65**	0.75**	
Crumb grain score	0.34	-0.46*	0.28	0.28	0.63**

*, ** significantly different from zero at 0.05 and 0.01 probability levels, respectively

Due to the differences in flour protein it was difficult to determine if the decreased loaf volume observed in the transgenic groups was the result of increased puroindoline protein content or decreased flour protein content. To minimize the effect of differing flour protein levels one line from each transgenic group and Hi-Line were milled as whole-wheat and then baked. Differences in flour protein of whole-wheat lines were minimal and did not differ significantly from each other (Table 10). Even with similar flour protein HGA-13 and HGAB-18 still exhibited significantly lower loaf volumes compared to Hi-Line (Table 10). HGB-6 exhibited no change in loaf volume but had a poorer crumb grain score than Hi-Line (Table 10). HGAB-18 had the poorest crumb grain score, while HGA-3 showed no change in crumb grain compared to Hi-Line (Table 10).

Table 10. Whole wheat bread baking traits for controls and transgenic lines based on mean of two replications from irrigated environment.

ID[a]	Flour protein	Flour ash	Mixograph absorption	Mix time	Loaf volume	Crumb Grain[b]
	———	g kg^{-1}	———	min	cc	
Hi-Line	151	17.3	624	2.90	770	3.50
HGA-13	150	18.0	621	3.20	693*	3.50
HGB-6	152	17.2	633	2.70	768	3.00
HGAB-18	154	18.0	624	3.05	643*	2.50
LSD(0.05)[c]	5.3	0.90	44.9	0.38	74.9	1.13

* significantly differ from Hi-Line at the 0.05 probability level.
a- All homozygous T_3 lines were derived from the hard spring wheat Hi-Line, which carries the *pina-D1a* and *pinb-D1b* alleles.
b- Based on 0 (unsatisfactory) to 5 (excellent) scale
c- LSD denotes least significant difference

Based on bake data from Quadrumat milled lines (Table 7) and whole-wheat lines (Table 10) it appears that high levels of puroindolines *in vivo* negatively affects both crumb grain score and loaf volume. In further support of this there were significant positive correlations found between SKCS hardness vs. loaf volume (r = 0.43), and SKCS hardness vs. crumb grain score (r = 0.39) (Table 8). In this experiment changes in SKCS hardness were a direct result of changes in both puroindoline sequence type and puroindoline content. These results conflict with previous findings which reported an improvement in crumb grain score and an increase in loaf volume after the reconstitution of blended flour with PINA (Dubriel et al 1998).

We were also interested in comparing the soft textured transgenic line HGAB-18 to Hi-Line when both were milled at a semi-commercial scale. To do so we implemented the use of a Miag Multomat pilot scale mill (Langerringen, Germany) which generated 14 mill streams per sample. Each stream was analyzed for ash (g kg^{-1}) (Figure 10) and protein content (g kg^{-1}) (Figure 11), which were then plotted against cumulative products

(%). Ash content increased slowly until approximately 70% product, but increased sharply beyond that point for both genotypes. The observed relationship is because the flour is milled from the inside to outside of the kernel. As total product increases ash also increases because the outer portions of the endosperm are high in ash. The two ash curves were similar in shape for the two genotypes, with Hi-Line having more ash content than HGAB-18 until about 70% product where the two genotypes switched ranks. This would indicate that the two genotypes differ in relative amounts of ash throughout the kernel. The protein curves showed a steady increase in protein with increasing cumulative product beyond about 30% product representing the increase in protein content of streams from the center of the kernel to streams closer to the aleurone layer and bran coat. The increase in protein was greater for HGAB-18 than for Hi-Line, with HGAB-18 having lower protein than Hi-Line at low level of product but higher protein than Hi-Line beyond about 60% product. This would indicate the two lines differ in distribution of protein from the center to the outside of the endosperm.

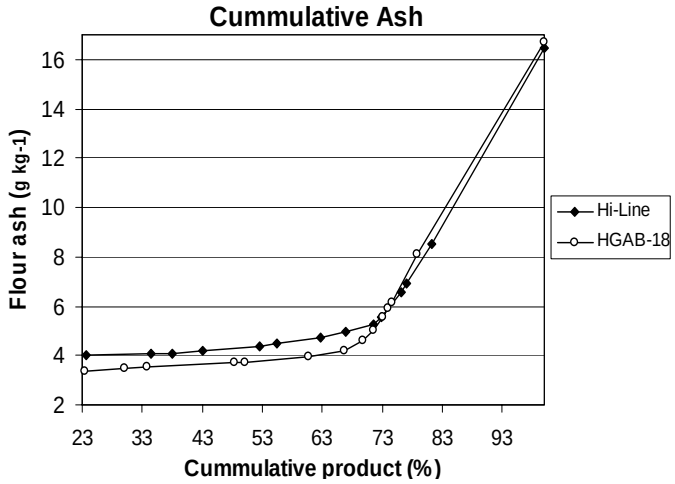

Figure 10. Cumulative ash curve for Hi-line (-•-) and HGAB-18 (-o-). The five highest points on each curve represent feed streams and are not incorporated into straight-grade flour.

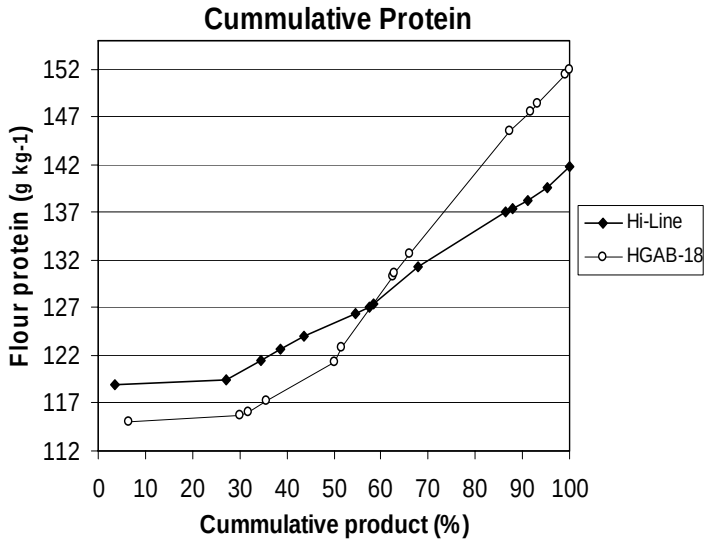

Figure 11. Cumulative protein curve for Hi-line (-•-) and HGAB-18 (-o-). The five highest points on each curve represent feed streams and are not incorporated into straight-grade flour.

The milling industry would prefer a wheat genotype that produces less ash at a given flour yield. Based on this criterion, HGAB-18 has superior milling characteristics compared to Hi-Line. "Straight-grade" flour yield for both Hi-Line and HGAB-18 was 715 g kg^{-1}. However, HGAB-18 had a slightly greater patent flour yield than Hi-Line, 696 vs. 673 g kg^{-1} respectively, and a much greater break flour yield than Hi-Line, 397 vs. 190 g kg^{-1} respectively. The increased patent and break flour yields reflect that HGAB-18 is much softer than Hi-Line. "Straight-grade" flour from HGAB-18 also had a lower ash content (5.3 vs. 5.9 g kg^{-1}) and a higher protein content (138 vs. 132 g kg^{-1}) compared to Hi-Line. Desirable characteristics being good flour yield, lower ash, and higher protein make HGAB-18 superior compared to Hi-Line, but the decreased starch damage in HGAB-18 flour would likely affect several of its rheological properties.

Here we have demonstrated that the over-expression of *pina-D1a* and *pinb-D1a* in the wheat seed endosperm not only causes drastic changes in grain texture but also affects a wide range of milling and bread baking traits. Almost every aspect of the milling process is affected by grain texture, which in turn is a direct manifestation of puroindoline allele type and quantity. Very high levels of puroindolines *in vivo* also negatively affects bread baking traits such as loaf volume and crumb grain score. By fully understanding how the puroindolines exert their influence over grain hardness and milling traits, breeders can then begin to utilize the puroindolines in creating optimal grain textures for millers that could yield advantages such as the ones demonstrated here on a semi-commercial scale.

CHAPTER 3

CONCLUSIONS

The underlying factors controlling wheat grain hardness are of great interest and have generated much controversy over the last century due to wheats importance as a food. As of recent researchers have molecularly characterized the genes *pina* and *pinb* (Gautier et al. 1994; Sourdille et al. 1996; Giroux and Morris 1997; Tranquilli et al. 1999), and their gene products which are directly involved in controlling wheat grain hardness. A well accepted hypothesis is that both functional PINA and PINB proteins, i.e. friabilin, must be present to create a soft phenotype in hexaploid wheat. When either PINA or PINB is missing or mutated, a hard phenotype is manifested. To further understand how this genetic system underlying wheat grain hardness works a hard spring wheat cultivar, Hi-Line, was genetically transformed with wild-type *pina*, *pinb*, or both. Hi-Line carries the common *pinb-D1b* mutation, which results in a Glycine-to-Serine change at the 46th residue of the mature PINB peptide. After using several molecular and physical techniques to characterize transformed lines some general conclusions can be drawn about *pina's* and *pinb's* role in controlling grain hardness.

Lines over-expressing either *pinb*, or *pina* and *pinb*, had significant decreases in grain harness which was the result of the genetic complementation of the mutated *pinb-D1b* allele in Hi-Line. The line over-expressing both puroindoline genes were the softest in texture. In lines that only over-expressed *pina* there was a decrease in grain hardness, but it was intermediate compared to that observed in lines transformed with

pinb, or *pina* and *pinb*. The intermediate decrease in hardness in the *pina* lines might be associated with the presence of the native, mutated PIN-D1B peptide, which could possibly retain some functionality. The over-expressing *pina* lines also lacked the biochemical marker friabilin, which is indicative of soft wheats. These results indicate that wheat grain hardness is not correlated with total puroindoline content but instead with the presence of functional PINA and PINB peptides. Changes in wheat grain hardness during seed development were also correlated with the expression of native and transgenic puroindolines, and the accumulation of friabilin.

The over-expression of the puroindolines in Hi-Line not only affected grain hardness but also affected several milling and bread baking characteristics. In all lines over-expressing the puroindolines there was a decrease in flour yield and an increase in break flour yield. Transgenic puroindoline lines also had significantly less flour ash and protein compared to the hard wheat control lines. Significant decreases in bread loaf volume and crumb grain score were also associated with the over-expression of puroindolines in the endosperm. When milled at a semi-commercial scale a line transformed with *pina* and *pinb* performed "better" than Hi-Line. At the same total flour yield flour the transgenic line had increased protein content, break flour yield, and patent flour yield, along with a decrease in ash content.

These findings support the hypothesis that *pina* and *pinb* are the primary genes controlling grain hardness in bread wheat. They also further the concept that a typical soft wheat phenotype can not occur unless both functional PINA and PINB proteins are

present during seed development. Lastly, changes in puroindoline expression and accumulation can influence a variety of milling and bread baking traits.

REFERENCES CITED

1. AACC. 2000. Approved methods of the American association of cereal chemists, 10th edition. American Association of Cereal Chemistry, St. Paul, Minnesota.

2. Baker R.J. 1977. Inheritance of kernel hardness in spring wheat. Crop Sci. 17:960-962.

3. Barlow K.K., M.M. Buttrose, D.H. Simmonds, and M. Vesk. 1973. The nature of the starch-protein interface in wheat endosperm. Cereal Chem. 50:443-454.

4. Bechtel D.B., J.D. Wilson, and C.R. Martin. 1996. Determining endosperm texture of developing hard and soft red winter wheats dried by different methods using the single-kernel wheat characterization system. Cereal Chem. 73:567-570.

5. Beecher B., A. Bettge, E. Smidansky, and M.J. Giroux. 2002. Expression of wild-type *pinb* sequence in transgenic wheat complements a hard phenotype. Theor. Appl. Genet. 105:870-877.

6. Bettge A.D., C.F. Morris, and G.A. Greenblatt. 1995. Assessing genotypic softness in single wheat kernels using starch granule-associated friabilin as a biochemical marker. Euphytica 86:65-72.

7. Blechl A.E. and O.D. Anderson. 1996. Expression of a novel high-molecular weight glutenin subunit gene in transgenic wheat. Nature Biotechnol. 14:875-879.

8. Blochet J.E., C. Chevalier, E. Forest, E. Pebay-Peyroula, M.F. Gautier, P. Joudrier, M. Pezolet, and D. Marion. 1993. Complete amino acid sequence of puroindoline, a new basic and cystine rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS Lett. 329:336-340.

9. Campbell K.G., C.J. Bergman, D.G. Gaulberto, J.A. Anderson, M.J. Giroux, G. Hareland, R.G. Fulcher, M.E. Sorrells, and P.L. Finney. 1999. Quantitative trait loci associated with kernel traits in a soft X hard wheat cross. Crop Sci 39:1184-1195.

10. Campbell K.G., P.L. Finney, C.J. Bergman, D.G.Gualberto, J.A. Anderson, M.J. Giroux, D. Siritunga, J. Zhu, F. Gendre, C. Roué, A. Vérel, and M.E. Sorrells. 2001. Quantitative trait loci associated with milling and baking quality in a soft x hard wheat cross. Crop Sci. 41:1275-1285.

11. Cane K., M. Spackman, and H.A. Eagles. 2004. Puroindoline genes and their effects on grain quality traits in southern Australian wheat cultivars. Aust. J. Agric. Res. 55:89-95.

12. Dubreil L., S. Meliande, H. Chiron, J.P. Compoint, L. Quillien, G. Branlard, and D. Marion. 1998. Effect of puroindolines on the breadmaking properties of wheat flour. Cereal Chem. 75:222-229.

13. Gautier M.F., M.E. Aleman, A. Guirao, D. Marion, and P. Joudier. 1994. *Triticum aestivum* puroindolines, two basic cystine-rich seed proteins: cDNA analysis and developmental gene expression. Plant Mol. Biol. 25:43-57.

14. Gautier M.F., P. Cosson, A. Guirao, R. Alary, and P. Joudrier. 2000. *Puroindoline* genes are highly conserved in diploid ancestor wheat and related species but absent in tetraploid *Triticum* species. Plant Sci. 153:81-91.

15. Giroux M.J. and C.F. Morris. 1997. A glycine to serine change in *puroindoline b* is associated with wheat grain hardness and low levels of starch-surface friabilin. Theor. Appl. Genet. 95:857-864.

16. Giroux M.J. and C.F. Morris. 1998. Wheat grain hardness results from highly conserved mutations in the friabilin components, *puroindoline a* and *b*. Proc. Natl. Acad. Sci. USA 95:6262-6266.

17. Giroux M.J., L. Talbert, D.K. Habernicht, S. Lanning, A. Hemphill, and J.M. Martin. 2000. Association of *puroindoline* sequence type and grain hardness in hard red spring wheat. Crop Sci 40:370-374.

18. Giroux M.J., T. Sripo, S. Gerhardt, and J.E. Sherwood. 2003. Puroindolines: Their role in grain hardness and plant defense. Biotechnology and Genetic Engineering Reviews Vol 20: in press.

19. Greenwell P. and J.D. Schofield. 1986. A starch granule protein associated with endosperm softness in wheat. Cereal Chem. 63:379-380.

20. Hogg A.C., T. Sripo, B. Beecher, J.M. Martin, and M.J. Giroux. 2004. Wheat puroindolines interact to form friabilin and control grain hardness. Theo. Appl. Genet. (in press)

21. Jeffers H.C. and G.L. Rubenthaler. 1977. Effects of roll temperature on flour yield with the Brabender Quadrumat Experimental mills. Cereal Chem. 54:1018-1025.

22. Jolly C.J., S. Rahman, A.A. Korrt, and T.J.V. Higgins. 1993. Characterization of the wheat Mr 15000 'grain softness protein' and analysis of the relationship between its accumulation in the whole seed and grain softness. Theor. Appl. Genet. 86:589-597.

23. Jolly C.J., G.M. Glenn, S. Rahman. 1996. GSP-1 genes are linked to the grain hardness locus (*Ha*) on wheat chromosome 5D. Proc Natl Acad Sci USA 93:2408-2413.

24. Krishnamurthy K. and M.J. Giroux. 2001. Expression of wheat *puroindolines* genes in transgenic rice enhances grain softness. Nature 19:162-166.

25. Krishnamurthy K., C. Balconi, J.E. Sherwood, and M.J. Giroux M.J. 2001. Increased tolerance to fungal diseases of rice plants transformed with *puroindoline* genes. Molec. Plant-Microbe Interact 13:1255-1260.

26. Lanning S.P., L.E. Talbert, F.H. McNeal, W.L. Alexander, C.F. McGuire, H. Bowman., G. Carlson, G. Jackson, J. Eckhoff, G. Kushnak, V. Stewart, and G. Stallknecht. 1992. Registration of Hi-line wheat. Crop Sci. 32:283-284.

27. Law C.N., C.F. Young, J.W.S. Brown, J.W. Snape, and J.W. Worland. 1978. The study of grain protein control in wheat using whole chromosome substitution lines. In: Seed Protein Improvement by Nuclear Techniques, International Atomic Agency, Vienna, Austria pp. 483-502.

28. Lillemo M. and C.F. Morris. 2000. A leucine to proline mutation in *puroindoline b* is frequently present in hard wheats from Northern Europe. Theor. Appl. Genet. 100:1100-1107.

29. Marion D., M.F. Gautier, P. Joudrier, M. Ptak, M. Pezolet, E. Forest, D.C. Clark, and W. Broekaert. 1994. Structure and function of wheat lipid binding proteins. In "Wheat Kernel Proteins-Molecular and Functional Aspects". Universita Degli Studi Della Tuscia. S Martino al Cimino, Viterbo (Italy) September 28-30, pp 175-180.

30. Martin J.M., R.C. Frohberg, C.F. Morris, L.E. Talbert, and M.J. Giroux. 2001. Milling and bread baking traits associated with *puroindoline* sequence type in hard red spring wheat. Crop Sci. 41:228-234.

31. Mattern J., R. Morris, J.W. Schmidt, and V.A. Johnson. 1973. Location of genes for kernel properties in the wheat cultivar 'Cheyenne' using chromosome substitution lines. In "Proceedings of the 4th International Wheat Genetics Symposium". University of Missouri, Columbia, Missouri, pp 703-707.

32. Morris C.F., G.A. Greenblatt, A.D. Bettge, and H.I. Malkawi. 1994. Isolation and characterization of multiple forms of friabilin. J. Cereal Sci. 21:167-174.

33. Morris C.F. and S.P. Rose. 1996. Chapter 1. Wheat. In “Cereal Grain Quality”. Chapman and Hall, New York, pp 3-54.

34. Morris C.F., M. Lillemo, G.M. Simeon, M.J. Giroux, S. Babb, and K. Kidwell. 2001. Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci. 41:218-228.

35. Ohm J.B., O.K. Chung, and C.W. Deyoe. 1998. Single-kernel characteristics of hard winter wheats in relation to milling and baking quality. Cereal Chem. 75(1):156-161.

36. Pomeranz Y., H. Boiling, and H. Zwingelberg. 1984. Wheat hardness and baking properties of wheat flour. J. Cereal Sci. 2:137-143.

37. Pomeranz Y., C.J. Peterson, and P.J. Mattern. 1985. Hardness of winter wheats grown under widely different climate condition. Cereal Chem. 62:463-467.

38. Riede C.R. and J.A. Anderson. 1996. Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci. 36:905-909.

39. SAS Institute Inc. 1988. SAS/STAT Users guide, Release 6.03 ed., Cary, NC. 1028 p.

40. Sivamani E., C.W. Brey, W.E. Dyer, L.E. Talbert, and R. Qu. 2000. Resistance to wheat streak mosaic virus in transgenic wheat expressing the viral replicase (*NIb*) gene. Mol. Breeding 6:469-477.

41. Slaughter D.C., K.H. Norris, and W.R. Hruschka. 1992. Quality and classification of hard red wheat. Cereal Chem. 69:428-432.

42. Sourdille P., M.R. Perretant, G. Charmet, P. Leroy, M.F. Gautier, P. Joudrier, J.C. Nelson, M.E. Sorrells, and M. Bernard. 1996. Linkage between RFLP markers and genes affecting kernel hardness in wheat. Theor. Appl. Genet. 93:580-586.

43. Sulaiman B.D. and R.W. Morrison. 1990. Proteins associated with the surface of wheat starch granules purified by centrifuging through caesium chloride. J. Cereal Sci. 12:53-61.

44. Symes K.J. 1965. The inheritance of grain hardness in wheat as measured by particle size index. Aust. J. Agric. Res. 16:113-123.

45. Symes K.J. 1969. Influence of a gene causing hardness on the milling and baking quality of two wheats. Aust. J. Agric. Res. 20:971-979.

46. Tranquilli G., D. Lijavetzky, G. Muzzi, and J. Dubcovsky. 1999. Genetic and physical characterization of grain texture-related loci in diploid wheat. Molecular and General Genetics 262:846-850.

47. Turnbull K.M., M. Turner, Y. Mukai, M. Yamamoto, M.K. Morell, R. Appels, and S. Rahman. 2003. The organization of genes tightly linked to the *Ha* locus in Aegilops tauschii, the D-genome donor to wheat. Genome 46: 330-338.

APPENDIX

2003 Dry Land Field Data

					SKCS[b]		
ID[a]	Flowering Date	Plant Height	Plot weight	NIR Protein	Hardness	Weight	Diameter
		inches	g	g kg^{-1}		mg	mm
Hi-Line	7/7	27.5	1114	181	82.4	25.3	2.13
161	7/7	29.0	1050	185	80.4	26.6	2.20
HWC[c]	**7/7**	**28.3**	**1082**	**183**	**81.4**	**25.92**	**2.16**
HGA 1[d]	7/7	26.3	920	179	58.0	27.5	2.19
HGA 3	7/7	29.0	1174	181	46.3	25.5	2.02
HGA 5	7/8	28.3	1003	190	49.5	25.0	2.03
HGA 13[d]	7/8	26.3	715	181	51.1	26.6	2.08
HGA AVG	**7/8**	**27.4**	**1088**	**182**	**51.2**	**26.2**	**2.08**
HGB 2	7/8	26.5	880	195	34.4	24.8	1.95
HGB 5	7/7	28.0	1070	193	28.9	23.8	1.91
HGB 6	7/7	27.3	900	182	24.8	25.2	1.98
HGB 12	7/7	31.0	1370	168	20.7	25.2	1.95
HGB 16	7/9	33.5	801	186	34.8	23.0	1.92
HGB 19	7/9	31.3	1633	174	23.6	25.6	1.97
HGB 21	7/7	29.0	1221	177	31.4	27.7	2.09
HGB 35	7/7	27.3	1044	191	28.0	23.0	1.90
HGB AVG	**7/7**	**28.6**	**997**	**187**	**29.0**	**24.6**	**1.95**
HGAB 2[d]	7/10	23.0	597	206	28.2	22.9	1.93
HGAB 3[d]	7/10	26.3	683	170	30.9	27.3	2.02
HGAB 11	7/7	28.3	974	181	25.6	23.8	1.88
HGAB 12	7/10	26.5	990	195	22.0	25.6	2.00
HGAB 18	7/7	27.8	1071	187	16.7	24.4	1.92
HGAB AVG	**7/9**	**26.4**	**1012**	**188**	**24.7**	**24.8**	**1.95**
LSD (0.05)[e]	2.11	2.65	336	10.9	4.54	2.00	0.12
HWC vs. HGA[f]	0.2189	0.2873	0.9560	0.8409	<.0001	0.6688	0.0205
HWC vs. HGB[f]	0.0781	0.3098	0.9557	0.4247	<.0001	0.0357	<.0001
HWC vs. HGAB[f]	0.0007	0.0136	0.4925	0.1148	<.0001	0.0468	<.0001
HGA vs. HGB[f]	0.6356	0.0146	0.9082	0.2396	<.0001	0.0043	<.0001
HGA vs. HGAB[f]	0.0080	0.0985	0.4907	0.0472	<.0001	0.0081	<.0001
HGB vs. HGAB[f]	0.0074	<.0001	0.4228	0.2461	<.0001	0.9606	0.5845

a- All homozygous T_3 lines were derived from the hard spring wheat Hi-Line, which carries the *pina-D1a* and *pinb-D1b* alleles

b- SKCS denotes Single kernel characterization system

c- HWC denotes average of hard wheat controls (Hi-Line and 161)

d- Were space seeded and are not included in the group plot weight average

e- LSD denotes least significant differences and values are used to compare individual transgenic lines vs. Hi-Line

f- For group comparisons p< values are given

2003 Irrigated Field Data

					SKCS[b]		
ID[a]	Flowering Date	Plant Height	Plot weight	NIR Protein	Hardness	Weight	Diameter
	Day in July	inches	g	g kg^{-1}		mg	mm
Hi-Line	7/8	33.1	1941	159	87.5	28.3	2.31
161	7/9	33.8	2208	159	84.1	30.48	2.46
HWC[c]	**7/8**	**33.4**	**2074**	**159**	**85.8**	**29.4**	**2.38**
HGA 1[d]	7/8	30.8	1548	160	55.2	29.2	2.30
HGA 3	7/7	32.3	1970	159	50.6	28.6	2.23
HGA 5	7/9	33.3	2003	170	48.1	27.0	2.16
HGA 13[d]	7/11	32.5	1499	161	56.1	29.6	2.30
HGA AVG	**7/8**	**32.2**	**1987**	**162**	**52.5**	**28.6**	**2.25**
HGB 2	7/11	32.0	1958	169	39.8	28.1	2.20
HGB 5	7/9	33.5	1872	167	27.5	27.2	2.12
HGB 6	7/7	33.3	1661	162	21.4	28.6	2.24
HGB 12	7/9	33.8	2004	161	18.7	27.8	2.15
HGB 16	7/9	43.3	1355	171	36.7	24.4	2.02
HGB 19	7/9	31.8	1376	176	20.9	25.4	2.03
HGB 21	7/7	32.0	1985	159	38.8	28.0	2.17
HGB 35	7/8	33.0	1934	168	25.1	26.1	2.06
HGB AVG	**7/8**	**34.4**	**1849**	**164**	**28.7**	**27.1**	**2.13**
HGAB 2[d]	7/11	29.3	1354	188	27.9	24.6	2.05
HGAB 3[d]	7/11	31.0	1591	151	24.3	29.1	2.12
HGAB 11	7/8	34.5	1864	157	16.6	26.0	2.02
HGAB 12	7/13	33.0	1779	170	27.0	24.4	2.00
HGAB 18	7/8	32.8	2171	164	13.1	26.9	2.08
HGAB AVG	**7/10**	**32.1**	**1938**	**166**	**21.8**	**26.2**	**2.06**
LSD (0.05)[e]	1.11	2.72	433	7.64	2.87	1.99	0.12
HWC vs. HGA[f]	0.4321	0.1156	0.5945	0.1439	<.0001	0.1650	0.0003
HWC vs. HGB[f]	0.5789	0.3674	0.0090	0.0007	<.0001	<.0001	<.0001
HWC vs. HGAB[f]	<.0001	0.0809	0.3036	0.0028	<.0001	<.0001	<.0001
HGA vs. HGB[f]	0.6994	0.0037	0.0834	0.0193	<.0001	0.0006	<.0001
HGA vs. HGAB[f]	<.0001	0.8942	0.7319	0.0616	<.0001	<.0001	<.0001
HGB vs. HGAB[f]	<.0001	0.0014	0.1138	0.7232	<.0001	0.0923	0.0105

a- All homozygous T_3 lines were derived from the hard spring wheat Hi-Line, which carries the *pina-D1a* and *pinb-D1b* alleles

b- SKCS denotes Single kernel characterization system

c- HWC denotes average of hard wheat controls (Hi-Line and 161)

d- Were space seeded and are not included in the HGA plot weight average

e- LSD denotes least significant differences and values are used to compare individual transgenic lines vs. Hi-Line

f- For group comparisons p< values are given