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ORIGINAL PAPER

A. C. Hogg · T. Sripo · B. Beecher · J. M. Martin · M. J. Giroux

Wheat puroindolines interact to form friabilin and control wheat grain hardness

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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, our objective was to identify and characterize the effect the *puroindoline* genes have on grain texture independently and together. To accomplish this we transformed a hard red spring wheat possessing a *pinb-D1b* mutation 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 puroindoline genes. The addition of PINA decreased grain hardness less than the reduction seen with added PINB. Seeds from lines having more 'soft type' PINB than PINA were the softest. Friabilin abundance was correlated with the presence of both 'soft type' PINA and PINB and did not correlate well with total puroindoline abundance. The data indicates that PINA and PINB interact to form friabilin and together affect wheat grain texture.

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A. C. Hogg · T. Sripo · B. Beecher · J. M. Martin · M. J. Giroux (✉)
Department of Plant Science and Plant Pathology,
Agriculture and Biological Sciences Faculty,
Montana State University-Bozeman,
Bozeman, MT 59717-3150, USA
e-mail: mgiroux@montana.edu
Tel.: +1-406-9947877
Fax: +1-406-9947600

Present address:
Division of Human Genetics, Department of Pathology,
Faculty of Medicine,
Prince of Songkla University,
Hatyai, Songkla, 90112, Thailand

Present address:
B. Beecher Institute of Agriculture and Natural Resources,
University of Nebraska-Lincoln,
152 Keim Hall, Lincoln, NE 68583-0915, USA

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 and Schofield 1986). It was later shown that genes encoding friabilin were closely linked to the *Ha* locus (Jolly et al. 1993, 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 and 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 and 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. This unique set of lines allowed us to determine if grain hardness is controlled by *pina*, *pinb*, or both. In all transformed lines there was a decrease in grain hardness observed. This change in grain texture was not correlated with total puroindoline amount, but instead the presence of both 'soft type' PINA and PINB.

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 residue 46 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 8 in 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.

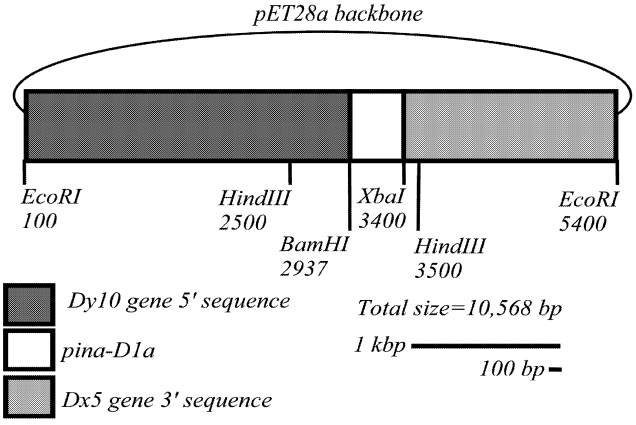

Fig. 1 *Pina* expression vector. *Above* is a schematic of the *pina* expression vector, pGA1.8. The glutenin coding region of the pGlu10(5) construct (Blechl and Anderson 1996) has been replaced with the 'soft-type' *pina* coding sequence, which was amplified from 'Chinese Spring' genomic DNA. The 'soft type' *pina* sequence is under the control of the glutenin regulatory elements *Dy10* (5′) and *Dx5* (3′)

Constructs

The *pina* expression vector, pGA1.8 (Fig. 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*AACAATGAAGGCCCTCTTCCTCATAGG3′) and AXP3 (5′AACTGCAG*TCTAGA*TCATCACCAGTAATAGCCAATA3′) were used to amplify the 'soft type' *pina* sequence, *pina*-D1*a*, from a 'Chinese Spring' genomic DNA extract (Reide and Anderson 1996) using *Taq* DNA polymerase (Promega, Madison, Wis.). These primers included the addition of *Bam*HI and *Xba*I sequences which are italicized above. The cycling parameters consisted of an initial denaturation step at 94ºC for 3 min, followed by 40 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 5 min. The amplified product was digested with *Bam*HI and *Xba*I, 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, Calif.).

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, Del.).

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′ATGAAGGCCCTCTTCCTCA3′) (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 bp 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) to individual leaves of plants using a cotton swab, which were then scored as either resistant or susceptible after 7 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) during the two-leaf stage of growth. After 7 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 12 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) 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 5 min at 13,000 *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, after which the sample was vortexed until homogenous, and then centrifuged as described. 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 as before. Next, 0.6 ml of the 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 1 h at −80°C. Samples were then centrifuged for 20 min at 13,000 *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, Minn.). 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 0.5*x*, 1*x*, 2*x*, 4*x*, and 6*x* total RNA concentration levels, where 1*x* 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 1*x* to 8*x*, in multiples of 1*x*, was used. This scale was constructed using a 'Hi-Line' TX-114 protein extract where 1*x* 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, Ill.) 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 *g* for 3 min. The CsCl was decanted and the starch was washed with 1 ml water. The starch water solution was vortexed until homogenous and then re-centrifuged. 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 3 min at 13,000 *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 −20°C, centrifuged for 3 min at 13,000 *g*, and the supernatant was removed. To the pellet 400 μl of −20°C acetone was added, followed by vortexing and another overnight incubation at −20°C. The following day the samples were removed from the −20°C 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 70°C for 10 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, Calif.). 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 1*x* to 8*x*, in multiples of 1*x* was used. This scale was constructed using a 'Hi-Line' TX-114 protein extract where 1*x* 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, Ill.), 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 7 days. After 7 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 10 min Particle size index reported is the percentage of flour recovered that is 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. Line 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. The untransformed 'Hi-Line' progeny were all susceptible while all of the 161 progeny tested were resistant to the herbicide glufosinate. 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 (Fig. 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 an over 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

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] resistant/susceptible	TX-114 test[d] PINA /PINB
	pGA1.8	pGB4.20		
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*-D1*a* sequence and a mutated *pinb*-D1*b* 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 7 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

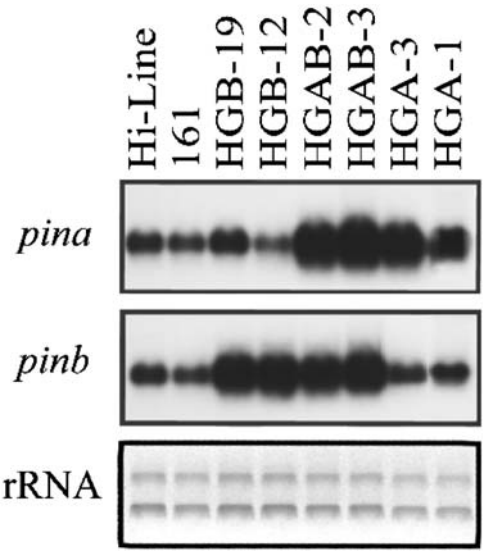

Fig. 2 *Puroindoline* expression analysis. Total RNA was extracted from immature kernels at 21 days post anthesis. Each lane was loaded with 1 μg of total RNA and replicate blots were probed with P^{32} labeled 'soft type' *pina* and *pinb* sequences. rRNA was used as a loading control and shows no sign 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

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.

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 *puroindoline* genes (Fig. 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 are no detectable transcripts of *pina* at 7 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. In both of the untransformed cultivars there are no *pinb* transcripts seen at 7 DPA, but in HGB-12 there is some accumulation of the 'soft type' *pinb* transcript. In HGB-12 total *pinb* transcript levels are highest at 14 DPA, which then progressively diminishes over time. In both 'Hi-Line' and 'Heron' a 'ramping-up' trend is 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.

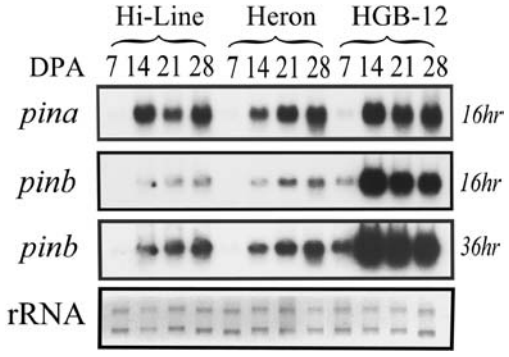

Fig. 3 Developmental *puroindoline* gene expression analysis. Total RNA was extracted from immature kernels at 7, 14, 21, and 28 days post anthesis. Each lane was loaded with 1 μg of total RNA and replicate blots were probed with P^{32} labeled 'soft type' *pina* and *pinb* sequences. rRNA was used as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

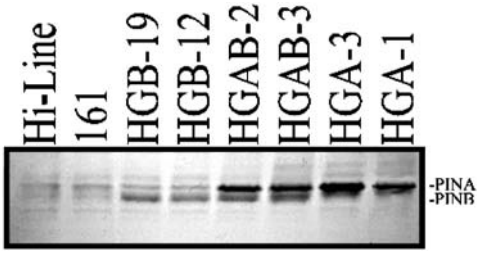

Fig. 4 Triton X-114 protein analysis. Using Triton X-114 detergent the puroindolines were extracted from 120 mg of coarsely milled whole-wheat flour. Proteins were fractionated via SDS-PAGE, and visualized using a Coomassie blue stain. The puroindolines can be resolved into two bands located around 15 kDa. In both control lines there are faint bands present for both PINA and PINB. In all transformed lines there is a considerable increase in total puroindoline abundance, especially the HGAB lines

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 (Fig. 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 (Table 2). '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

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 0.5*x* to 6*x* scale, where 1 μg=1*x*. 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 8*x*. 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 0.5*x* to 8*x*. Number is an average of two experiments

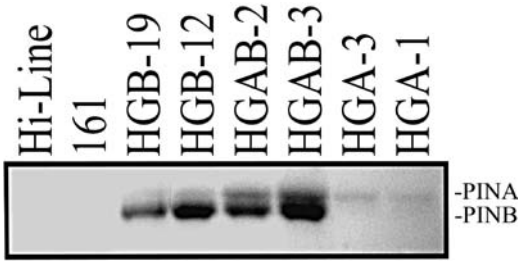

Fig. 5 Friabilin analysis. Friabilin was extracted off the surface of water-washed starch granules isolated from the selected transformed and control lines. Friabilin samples were fractionated using SDS-PAGE, and visualized using a Coomassie blue stain. The friabilin marker can be resolved into its component 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. This indicates that PINA/PINB interactions are required for friabilin formation

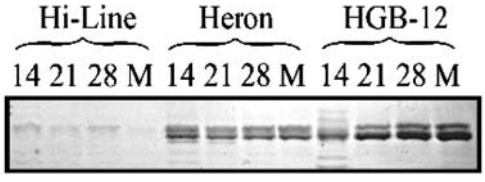

Fig. 6 Developmental friabilin analysis. Friabilin was extracted off the surface of water-washed starch granules isolated from 'Hi-Line', 'Heron', and HGB-12. Samples were collected over a developmental period of 3 weeks and at maturity. Seeds were air-dried and then milled into coarse whole-wheat flour. Friabilin extracts were fractionated using SDS-PAGE, and visualized using a Coomassie blue stain. For the hard spring wheat 'Hi-Line' there is no friabilin present while the soft spring wheat 'Heron' and the transformed line HGB-12 friabilin increases during maturity

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 (Table 2). HGB-12 has a 'soft type' PINB level equal to 4.0 and a PINA level equal to 1.0 (Table 2). For HGAB-2 there was an increase in both PINA and 'soft type' PINB with levels equal to 7.0 and 4.0 respectively (Table 2). 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 (Table 2). 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 (Table 2). Both of the HGA lines were given a 'soft type' PINB value of 0.0 (Table 2).

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 (Fig. 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 (Table 2). HGB-19 does show friabilin accumulation and its component proteins PINA and PINB have values of 1.0 and 4.0 respectively (Table 2). HGB-12 also shows friabilin accumulation with a PINA value of 1.5 and a PINB value of 6.0. Friabilin is observed as well in HGAB-2 and HGAB-3, which have PINA values equal to 4.0 and 6.0 and PINB values equal to 5.0 and 8.0 respectively (Table 2). HGA lines lack friabilin, with no band present for PINB and only a very faint band for PINA that is equal to 2.0 for both lines. This faint band for PINA is not observed in either of the control lines.

Friabilin was also extracted from 'Hi-Line', 'Heron', and HGB-12 kernel samples collected at 14, 21, and 28 DPA, and at maturity (Fig. 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.

Table 3 Grain hardness determined by single kernel characterization system (SKCS) analysis and particle size index for selected lines. All T_3 lines were derived from the spring wheat variety 'Hi-Line', which carries a 'soft type' *pina*-D1*a* sequence and a mutated *pinb*-D1*b* sequence. Homozygosity was confirmed using PCR, herbicide test, and amount of PIN in individual kernels. Single kernel hardness values are an average of four replications. Means followed by the same letter do not differ from each other based on Student's *T* test at the 0.05 probability level. The particle size index (PSI) was determined using a 90 μm screen and 10 g of coarsely milled whole wheat flour

Line	SKCS	PSI%
Hi-Line	67.5±3.8a	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

Table 4 Developmental hardness analysis of selected lines using SKCS

Line	DPA[b]	SKCS[c]	SD[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[a]	14	74.3	19.3
HGB-12[a]	21	49.6	12.9
HGB-12[a]	28	27.6	12.2
HGB-12[a]	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

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 an almost 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.

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 (Fig. 3) as well as friabilin accumulation (Fig. 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.

Discussion

Our objective was to identify and characterize the role of the puroindolines 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×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 *puroindoline* genes 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 *puroindoline* genes 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 *puroindoline* genes are the primary genetic elements

in determining grain hardness. In lines transformed with both *pina-D1a* and *pinb-D1a* sequences, and the *pinb-D1a* sequence alone, there were increased *puroindoline* transcript levels (Fig. 2), increased puroindoline content (Fig. 4), and a phenotype typical of soft wheats. This phenotype was characterized by the presence of the protein marker friabilin (Fig. 5), decreased grain hardness (Table 3), and decreased flour particle size (Table 3). In lines transformed exclusively with the *pina*-D1*a* sequence there was an increase in *pina* transcript levels (Fig. 2; Table 2) and PINA content (Fig. 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, 2). 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, correlating with the absence of friabilin (Fig. 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*-D1*a* and *pinb*-D1*a* 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 (Table 4; Fig. 3) and friabilin accumulation (Table 4; Fig. 6). 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.

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