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ORIGINAL PAPER

Complementation of the pina (null) allele with the wild type *Pina* sequence restores a soft phenotype in transgenic wheat

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Abstract The tightly linked puroindoline genes, *Pina* and *Pinb*, control grain texture in wheat, with wild type forms of both giving soft, and a sequence alteration affecting protein expression or function in either giving rise to hard wheat. Previous experiments have shown that addition of wild type *Pina* in the presence of mutated *Pinb* gave intermediate grain texture but addition of wild type *Pinb* gave soft grain. This raises questions as to whether *Pina* may be less functional than *Pinb*. Our goal here was to develop and characterize wheat lines expressing the wild type *Pina-D1a* sequence in hard wheat with the null mutation (*Pina-D1b*) for *Pina*. Three transgenic lines plus Bobwhite were evaluated in two environments. Grain texture, grain protein, and kernel weight were determined for the transgenic lines and Bobwhite. The three transgenic lines had soft phenotype, and none of the transgenic lines differed from Bobwhite for grain protein or kernel weight. The soft phenotype was accompanied by increases in *Pina* transcript accumulation. Total Triton X-114 extractable PINA and PINB increased from 2.5 to 5.5 times those from a soft wheat reference sample, and friabilin, PINA and PINB bound to starch, increased from 3.8 to 7.8 times those of the soft wheat reference. Bobwhite showed no starch bound PINA, but transgenic lines had levels from 5.3 to 13.7 times those of the soft wheat reference sample. Starch bound PINB in transgenic lines also increased from 0.9 to 2.5 times that for the soft wheat reference sample. The transgenic expression of wild type *Pina* sequence in the *Pina* null genotype gave soft grain with the characteristics of soft wheat including increased starch bound friabilin. The results support the hypothesis that both wild type *Pin* genes need to be present for friabilin formation and soft grain.

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Introduction

Wheat (*Triticum aestivum*) grain is classified as hard or soft based on the texture of the endosperm. These textural classes coincide with important differences in milling and end-use properties (Pomeranz and Williams 1990; Morris and Rose 1996). The distinction between soft and hard classes of wheat is governed mainly by the Hardness (*Ha*) locus on chromosome 5DS (Mattern et al. 1973; Law et al. 1978).

Greenwell and Schofield (1986) identified friabilin as a marker protein for grain softness present in large amounts on the surface of water-washed starch of soft class of wheat and nearly absent from hard class of wheat (Bettge et al. 1995; Greenblatt et al. 1995; Morris et al. 1994). Friabilin is composed primarily of two polypeptides termed puroindoline a (PINA) and puroindoline b (PINB). Genes coding for these two proteins, *Pina* and *Pinb*, are tightly linked to the *Ha* locus on chromosome 5D (Sourdille et al. 1996 ; Giroux and

Morris 1997). Recent results have shown that mutations in either *Pina* or *Pinb* are associated with hard texture (Giroux and Morris 1997, 1998; Lillemo and Morris 2000; Morris et al. 2001a, b).

Krishnamurthy and Giroux (2001) provided evidence that puroindoline genes are responsible for variation in grain texture by transgenically expressing wild type *Pina-D1a* and *Pinb-D1a* sequences in rice (*Oryza sativa*), which has no puroindoline homologue (Gautier et al. 2000). Grain from the transgenic plants had softer texture than untransformed controls. In wheat, transgenic expression of wild type *Pinb-D1a* sequence in the hard spring wheat 'Hi-Line', which contains the *Pinb* glycine to serine mutation (*Pinb-D1b* allele), complemented the *Pinb-D1b* allele resulting in a soft phenotype (Beecher et al. 2002). Hogg et al. (2004) expressed *Pina-D1a*, *Pinb-D1a*, or both in the same Hi-Line background. Expression of *Pinb* or both *Pina* and *Pinb* gave a soft phenotype, while adding *Pina* alone gave grain intermediate in texture. They concluded that both *Pina* and *Pinb* must be present to form friabilin resulting in the softest phenotype, but expression of *Pinb* may be more limiting than *Pina*.

The transformation experiments with Hi-Line implicate the role of both puroindolines in grain texture. Results from Hogg et al. (2004) raise questions of whether the intermediate texture found when *Pina-D1a* sequence was added to Hi-Line relative to the soft texture seen when *Pinb-D1a* sequence was added was the result of only wild type *Pina-D1a* without functional *Pinb* in Hi-Line or greater function of *Pinb*. Therefore, our goal here was to develop and characterize wheat lines expressing the wild type *Pina-D1a* sequence in 'Bobwhite', a hard wheat with the *Pina-D1b* null mutation.

Materials and methods

Wheat transformation and regeneration

The hard white spring wheat cultivar Bobwhite was transformed with *Pina* and *Bar* expression vectors. Bobwhite has puroindoline genotype *Pina-D1b/Pinb-D1a*, the former being a null allele for *Pina*. The pGA1.8 *Pina* expression vector was described in Hogg et al. (2004). In short, the *Pina-D1a* gene was placed between the Glutenin gene flanking regulatory sequences from pGlu10H5 (Blechl and Anderson 1996). The *Bar* expression vector was pAHC20 (Christensen and Quail 1996), which confers resistance to the herbicides bialaphos (Meiji Seika Kaisha Ltd, Japan) and glufosinate (AgrEvo USA Company, Wilmington, DE). The plasmids encoding PINA and BAR were introduced in a 3:1 molar ratio into immature embryos using Model PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA) as described by Blechl and Anderson (1996). After 4 weeks on non-selective media, transformed plants were regenerated from the calli under 1 mg/l bialaphos selection as described by Okubara et al. (2002). Transformation was verified by PCR analysis of genomic DNA prepared from T_0 and T_1 plants as described by Okubara et al. (2002), using primers in the Glutenin 5′ and 3′ regulatory sequences—5′GCTGAA CTAACTCGCCGTGC3′ and 5′GCCAGGGATATT GCCACACC3′ to amplify a 862 bp fragment that included the entire PINA coding sequence found within the construct. PCR amplification was carried out as in Okubara et al. (2002) except that the annealing temperature was 67.5°C.

Derivation of transgenic lines

T_1 seeds from three transformation events and Bobwhite were planted in the greenhouse, and 12–24 individual T_1 plants from each transformation event were subsequently harvested. Grain hardness was tested using the Perten Single Kernel Characterization System (SKCS) 4100 (Perten Interments, Springfield, IL) using 50 or more T_2 seeds from each T_1 plant. Increased SKCS grain hardness standard deviations are observed in samples of wheat grain in which the puroindoline genotype is not the same in each seed (Giroux and Morris 1998; Morris et al. 2001a, b). A T_1 plant was judged homozygous for the *Pina* transgene if seed from that plant had mean SKCS hardness less than Bobwhite, and the SKCS standard deviation was equal to or less than Bobwhite (<20), while T_1 plants were considered heterozygous for the *Pina* transgene if seed from that plant had SKCS standard deviation greater than 25. T_1 plants were judged homozygous for the absence of the *Pina* transgene if the SKCS hardness mean and standard deviation were similar to untransformed Bobwhite. T_2 seed from single T_1 plants believed to be homozygous for the *Pina* transgene, based on the criteria cited above, were planted in single 3 m rows with plants spaced 15 cm apart in the field during the 2004 growing season. Each of the three transformation events was represented by at least 3 T_1 derived T_2 rows with 50 or more plants per transformation event. Single T_2 plants were harvested and seed used for further progeny testing. Grain hardness phenotype was evaluated using 50 T_3 seed from each harvested T_2 plant. T_2 plants were judged homozygous or heterozygous for the *Pina* transgene using the same criteria as mentioned earlier. For each of the three events,

all T_2 plants grown in the field in the 2004 season had a low SKCS grain hardness mean and standard deviation typical of a homozygous soft wheat. Individual T_2 plants within each transformation event were also progeny tested for the presence of *Bar*. Eighteen T_3 seeds from individual T_2 plants from each event were planted with three seeds in each of six 25-cm^3 cells in a container with six cells per container. Plants were sprayed with 0.1% glufosinate solution at the two-leaf stage. Plants were scored as resistant or susceptible 5–7 days after spraying. Susceptible plants were characterized by wilting and chlorosis, while resistant plants remained green. T_2 plants giving progeny in which all sprayed plants were resistant were considered homozygous positive for *Bar*, and T_2 plants having progeny where all sprayed plants were herbicide susceptible were considered homozygous negative for *Bar*. Progeny with mixed herbicide results were considered derived from T_2 plants heterozygous for *Bar*.

Northern blot analysis

The protocol for Northern blot analysis was outlined in Hogg et al. (2004). Briefly, total RNA was extracted from 100 mg of frozen, ground seed tissue representing a pool of five or more T_3 seeds. Seeds were taken from homozygous T_2 plants 21 days post anthesis. All T_2 plants were homozygous positive for *Pina* (transgenic). Bobwhite and Heron plants were used as non-transgenic negative and positive reference samples, respectively for *Pina* expression. Heron is a soft wheat with wild type forms of *Pina* and *Pinb*. Heron RNA was loaded in a series of increasing amounts to account for varying signal intensities among the transgenic lines. All RNAs were loaded in duplicate (1 μg per well), separated on a 1.6% agarose gel, and blotted onto a nylon membrane. The membrane was cut to separate duplicate RNA loads and probed with either a ^{32}P-labeled *Pina* or a ^{32}P-labeled *Pinb* probe. Following hybridization, membranes were washed with increasing stringencies of SSPE, dried, and placed on film for several days. Specifically, there were two washes 30 min with 2× SSPE and two 30 min washes with 0.2× SSPE and all washes were at 67°C (Beecher et al. 2002). Membranes were re-probed with a ribosomal RNA probe after the PINA and PINB probes were removed to assess RNA quantity and quality.

Southern blot analysis

Genomic DNA was extracted (Riede and Anderson 1996) from composite samples representing five or more seedlings for Bobwhite and from a composite of five or more homozygous T_2 seedlings for each of the three *Pina* transgenic lines. Forty μg of genomic DNA was digested with *Bam*HI. *Bam*HI cuts the pGA1.8 vector just once and does not cut at all within the *Pina* coding sequence. Digests were loaded into two separate wells (20 μg DNA per well) and fractionated on a 0.8% agarose gel. Following capillary blot transfer, the nylon membrane was cut in half to separate duplicate DNA loads. Each membrane was incubated overnight with either a ^{32}P-labeled *Pina* or a ^{32}P-labeled *Pinb* probe. Following hybridization, membranes were washed and exposed to film as described above.

Triton X-114 protein extraction and western blot analysis

Total puroindoline was extracted using Triton X-114 (TX-114) detergent essentially as described by Giroux et al. (2003). Triton X-114 extractions were performed on the three transgenic lines, the soft wheat Heron and Bobwhite. As in Giroux et al. (2003) 100 mg of fine powder obtained by grinding mature seeds was extracted overnight with the detergent TX-114. Proteins in the detergent phase were precipitated with acetone, washed, dried, resuspended in SDS sample buffer (without BME), fractionated on a 10–20% Tris–HCl gel (Biorad, Hercules, CA), and stained with coomassie blue R250. Loads were 1, 2.5, 5 and 7*x*, where the *x* is the weight equivalent of input powder. Total TX-114 extractable PIN for the three transgenic lines and Bobwhite was quantified using Heron PIN content as a relative value of one. Two independent extractions were performed on each line. Following the TX-114 extractions, the proteins were then transferred to a nitrocellulose membrane and probed with a PINA polyclonal antibody as previously described (Krishnamurthy and Giroux 2001).

To further confirm the transgenic lines were homozygous for the *Pina* transgene, TX-114 protein extractions were performed on 12 random T_1 derived T_5 seeds of each transgenic line obtained after the 2005 growing season (see below) along with Bobwhite and Heron. The TX-114 proteins were then probed with the PINA polyclonal antibody described by Krishnamurthy and Giroux (2001). Seeds were considered positive for PINA (and the *Pina* transgene) if their TX-114 protein extracts contained a 14 kDa protein recognized by the PINA antibody. Heron is a soft wheat that contains *Pina-D1a* and *Pinb-D1a* (Giroux and Morris 1998) and so served as a PINA positive control. Bobwhite does not contain any PINA antibody reactive protein. T_1 derived T_5 seed pools were considered homozygous if all 12 seeds contained relatively equal amounts of PINA antibody reactive protein.

Friabilin protein extraction and analysis

Friabilin, or starch bound puroindoline, was isolated from the surface of starch granules using the same genotypes as for TX-114 protein extractions. The methods used were a combination of the single seed friabilin preparation described by Bettge et al. (1995) and the starch surface protein CsCl purification method of Sulaiman and Morrison (1990). Briefly, 70–150 mg of finely ground whole-wheat flour was suspended in water and centrifuged through 80% CsCl allowing starch granules to pellet. Purified starch granules were washed with water three times and once with acetone. The washed starch was then dried and weighed. Proteins bound to the surface of washed starch granules were extracted with 50% isopropanol/0.5 M NaCl, precipitated with acetone, pelleted by centrifugation, dried, suspended in SDS sample buffer in a proportion of 120 μl buffer/100 mg starch, fractionated on a 10–20% Tris–HCl gel (Biorad, Hercules, CA), and stained with coomassie blue R250. Loads were adjusted to make the signal strength from PINA and PINB, visible as distinct bands as indicated, similar enough to allow comparison of amounts of seed starch granule-bound PINA and PINB in different transgenic lines and controls. Total friabilin and its components, PINA and PINB, were quantified relative to Heron.

Field evaluation

The untransformed Bobwhite and T_1 derived T_4 homozygous transgenic lines were evaluated in a randomized block design with two replications during the 2005-growing season at Bozeman, MT. The same experiment was grown in irrigated and rain-fed conditions. Each plot was two 3 m rows spaced 30 cm apart with each row seeded with 5 g of seed using T_1 derived T_4 seeds. Plots were cut and the grain threshed with a Vogel thresher (Bill's Welding, Pullman, WA). Grain hardness and kernel weight were measured using the Perten Single Kernel Characterization System (SKCS) 4100 (Perten Instruments, Springfield, IL) using 300 seeds from each plot. Grain protein was determined on grain samples from each plot using an Infratec 1225 Grain Analyzer (Tecator Hoganas, Sweden) whole kernel analyzer.

Statistical analyses

Analysis of variance was computed for grain hardness, grain hardness standard deviation, grain protein, and kernel weight using a randomized block model combined over environments using PROC GLM in SAS (SAS Institute 2004). A least significant difference was computed, and genotypes were compared when the genotypes were different at $P < 0.05$.

Results

After transformation with the pGA1.8 *Pina* expression vector (Hogg et al. 2004), three transgenic Bobwhite lines were chosen for further propagation (Materials and methods) and detailed characterization. Phenotypic data for SKCS hardness using seed from T_4 plants grown in the field showed the three transgenic lines were all softer than the Bobwhite control, and the three transgenic lines could not be differentiated from

Table 1 Means for seed characteristics and glufosinate herbicide reaction of Bobwhite hard spring wheat and three Bobwhite transgenic lines co-transformed with *Pina* and *Bar* grown in irrigated and rain-fed environments at Bozeman, MT

Genotype[a]	*Bar*[b]	Grain hardness[d]	Grain hardness standard deviation[d]	Grain protein (%)	Kernel weight (mg)
Bobwhite	–	82.6	15.6	15.5	26.4
BW2	–	21.7	15.4	15.4	25.6
BW3	+	24.8	15.3	15.3	26.3
BW5	+	21.5	15.8	15.8	28.1
P Value[c]		0.000	0.112	0.452	0.064
LSD(0.05)		5.9	0.7	0.7	1.7
CV%		9.1	8.4	2.6	3.7

[a] BW2, BW3, and BW5 are independent Bobwhite transgenic lines expressing the wheat *Pina* coding sequence. Bobwhite is the untransformed hard parental control

[b] Bobwhite calli were co-bombarded with the *Bar* gene which confers resistance to the herbicide glufosinate. Lines designated as + demonstrated glufosinate resistance whereas lines shown as – were sensitive to glufosinate

[c] Probability of a larger value of *F* for differences among genotypes

[d] Obtained from single kernel characterization system

each other ($P < 0.05$) (Table 1). The four genotypes did not differ ($P < 0.05$) for mean SKCS standard deviation. The three transgenic lines met the initial criteria used to classify them for the presence of the *Pina* transgene (soft phenotype and SKCS standard deviation <20). Grain protein and individual seed weight showed no significant variation among Bobwhite and the transgenic lines. There was no evidence of genotype by environment interaction for grain hardness, grain protein or kernel weight ($P < 0.05$) (data not shown). Although Bobwhite calli were co-bombarded with wild type *Pina* sequence and *Bar*, which confers resistance to the herbicide glufosinate, one of the transgenic lines (BW2) was sensitive to glufosinate (Table 1). Western blotting of individual T_1 derived T_5 seeds from each of the three transgenic lines and controls showed the *Pina* transgene was expressed in all individual seeds in each transgenic line, verifying the lines were homozygous for the transgene (data not shown).

Southern blot analysis was performed to ensure that the three selected lines represented independent transformation events. Figure 1 demonstrates all lines hybridized with the *Pinb* probe with all showing the same banding pattern. Results using the *Pina* probe showed no distinct hybridizing bands in the Bobwhite control. The three transgenic lines displayed multiple bands with varying size. The unique banding patterns with multiple bands of varying size indicates the three lines represent independent transformation events with varying insertion sites and copy number.

Total RNA from wheat seeds 21 days post anthesis was analyzed to assess *Pin* transcript accumulation (Fig. 2). The soft wheat Heron, which expresses both native *Pina* and *Pinb*, was loaded in varying amounts

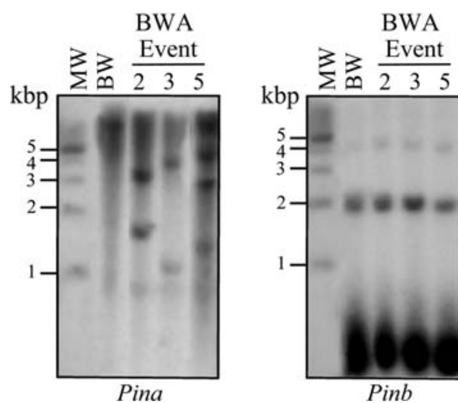

Fig. 1 Southern blot analysis for *Bam*HI digested genomic DNA for untransformed Bobwhite (*BW*) and homozygous *Pina* positive T_3 plants from three transformed lines (*BW2*, *BW3*, and *BW5*). *Bam*HI was cut once in the transformation vector. Each membrane was incubated with either a ^{32}P-labeled *Pina* or a ^{32}P-labeled *Pinb* probe

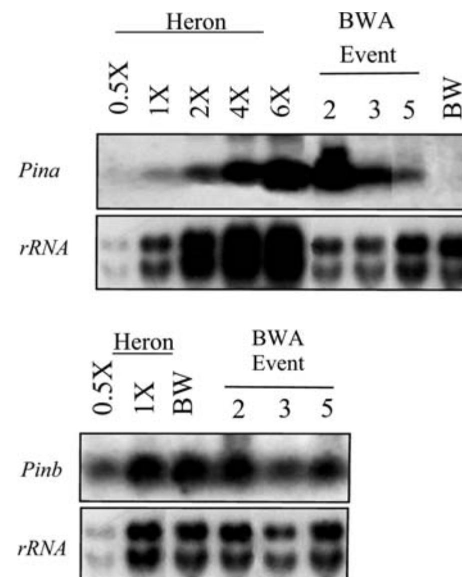

Fig. 2 Northern blot analysis of *Pina* and *Pinb* transcript accumulation for developing wheat T_3 seeds (21 days post anthesis) for three Bobwhite lines homozygous-positive for *Pina* sequence (*BW2*, *BW3*, and *BW5*), untransformed Bobwhite (*BW*), and soft wheat Heron loading control. RNAs were loaded in duplicate and separated on a 1.6% agarose denaturing gel. The membrane was cut to separate duplicate RNA loads and probed with either a ^{32}P-labeled *Pina* or a ^{32}P-labeled *Pinb* probe. Heron RNAs were loaded in a series of increasing amounts ($0.5x = 0.5$ μg, $1x = 1$ μg, etc.) to account for varying signal intensities among transformed lines

to serve as reference for the three transformants and Bobwhite. Bobwhite, lacking *Pina*, did not accumulate *Pina* transcript, whereas the transformants accumulate *Pina* transcript in varying amounts. BW5 appears to accumulate less *Pina* transcript than BW3, which has less than BW2. However, the relatively unequal loading evidenced by the unequal rRNA signals renders exact quantification difficult at best. As expected, Heron, Bobwhite and the three transformants all show similar levels of *Pinb* transcript accumulation.

We assessed the relative amounts of total PIN (PINA and PINB) and friabilin (starch-bound PINA and PINB) for the three Bobwhite transformants and untransformed Bobwhite compared to soft wheat Heron. Heron showed more total PIN than Bobwhite at comparable loads (Fig. 3), and relative amounts of total PIN varied across the three transgenic Bobwhite lines. Amounts of total PIN relative to Heron in transgenics varied from 2.5*x* for BW5 to 5.5*x* for BW2 (Table 2). Total PINA from TX-114 extraction was estimated using a Western blot for Heron, Bobwhite, and the three transgenic lines (Fig. 4). The three transgenic lines varied in total PINA relative to Heron from 1.6*x* (BW3) to 5*x* (BW2) (Table 2). The effect of the added *Pina* expression on starch-bound PINA and PINB, which comprise friabilin, was determined

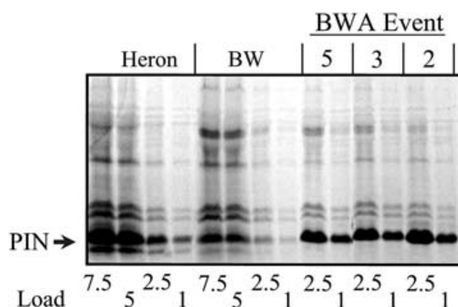

Fig. 3 SDS-PAGE gel of Triton X-114 soluble proteins from mature wheat seeds for three independent Bobwhite transgenic lines (*BW2*, *BW3*, and *BW5*) expressing *Pina* sequence and untransformed Bobwhite (BW) and Heron references. Loads were as indicated beneath the gel. The band labeled *PIN* consisted of both PINA and PINB. Independent extractions for each transgenic line are shown side by side

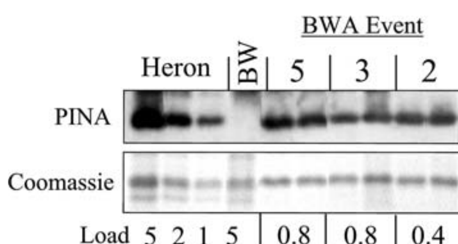

Fig. 4 Immunoblot comparison of puroindoline a (*PINA*) dosage in mature wheat seeds for three independent transgenic lines (BW2, BW3, and BW5) expressing *Pina* sequence and untransformed Bobwhite (*BW*) and Heron references. Loads for different transgenic lines and controls (indicated beneath *coomassie* panel) were adjusted to make the amount of PINA + PINB in each lane roughly equivalent (*coomassie* panel). Proteins were transferred to a nitrocellulose membrane and probed with PINA polyclonal antibody (*PINA* panel). Independent extractions for each transgenic line are shown side by side

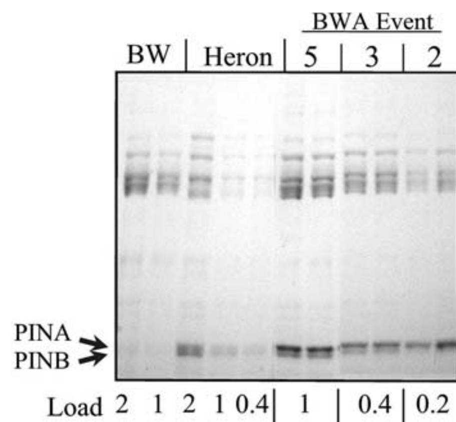

Fig. 5 SDS-PAGE gel of mature seed starch friabilin (starch granule-bound PINA and PINB) for three independent transgenic lines (*BW2*, *BW3*, and *BW5*) expressing *Pina* sequence and untransformed Bobwhite (BW) and Heron references. Loads (indicated beneath gel) were adjusted to make the signal strength from PINA and PINB visible as distinct bands as indicated, similar enough to allow comparison of amounts of seed starch granule-bound PINA and PINB in different transgenic lines and controls. Independent extractions for each transgenic line are shown side by side

(Fig. 5). Heron showed both PINA and PINB, while Bobwhite showed no PINA, and no detectable PINB at loading levels used in Fig. 5. The amounts of PINA and PINB were quantified relative to Heron. The three transgenic lines showed increased amounts of friabilin associated PINA and PINB (Table 2). These amounts ranged from 5.3*x* (BW5) to 13.7*x* (BW2) for PINA, and from 0.9*x* (BW2) to 2.5*x* (BW3) for PINB relative to Heron. Similar trends were observed for collective amounts of starch-bound PINA plus PINB, where all transgenic lines showed more total bound PIN than the soft wheat.

Discussion

Hard wheat grain phenotype results from a sequence alteration affecting protein expression of function in either *Pina* or *Pinb*. The *Pinb-D1b* allele, resulting from a glycine to serine change in *Pinb* is the most common mutation among hard wheat. A null allele for *Pina (Pina-D1b)* giving no *Pina* transcript is less frequent (Morris et al. 2001a, b). Amoroso et al. (2004) used RT-PCR to quantify *Pina* and *Pinb* expression in hard and soft wheat. Hard wheat with *Pina-D1b* allele did not accumulate *Pina*, but hard wheat with *Pinb-D1b* allele did accumulate *Pinb* transcript in variable amounts. Lines homozygous for *Pinb-D1b* allele averaged about six SKCS units softer than lines homozygous for *Pina-D1b* allele in a recombinant inbred population segregating for *Pina-D1b* and *Pinb-D1b* alleles (Martin et al. 2001). These reports indicate *Pinb-D1b* allele may maintain at least partial function. Alternatively, PINA may contribute more to grain softness than PINB. Clarke and Rahman (2005) used microarray analysis to show that *Pina* was more highly expressed than *Pinb* in soft versions of soft and hard isolines, which supports the hypothesis that *Pina* may be more important than *Pinb* in determining grain texture.

The parent cultivar Bobwhite used in this study has a hard texture phenotype and wild type *Pinb* (*Pinb-D1a* allele) and *Pina* null alleles (*Pina-D1b*) (Giroux and Morris 1998). Bobwhite was transformed with wild type *Pina* sequence. We have characterized three transgenic lines for the presence of the *Pina* sequence, transcript accumulation, and amounts of total and starch bound PINA and PINB. These lines were further evaluated for grain texture and other grain characteristics. Beecher et al. (2002) and Hogg et al. (2004)

Table 2 Puroindoline a (PINA) and puroindoline b (PINB) amounts in three Bobwhite *Pina* transgenic spring wheat lines relative to untransformed Bobwhite or soft wheat Heron

Genotype[a]	PINA + B TX-114 versus Heron[b]	PINA + B friabilin versus Heron[c]	PINA TX-114 western versus Heron[b]	PINA friabilin versus Heron[c]	PINB friabilin versus Heron[c]
BW2	5.5 (0.50)	7.8 (2.7)	5.0 (0)	13.7 (4.6)	0.9 (0.5)
BW3	3.2 (0.65)	4.8 (0)	1.6 (0.30)	6.7 (0)	2.5 (0)
BW5	2.5 (0)	3.8 (0)	2.2 (0.30)	5.3 (0)	2.0 (0)
Heron	1	1	1	1	1
Bobwhite	0.6	0.1	0	0	0

[a] BW2, BW3, and BW5 are independent Bobwhite transgenic lines expressing the wheat

Pina coding sequence. Heron is a type of untransformed soft spring wheat. Bobwhite is the untransformed hard parental control

[b] The Triton X-114 (TX-114) protocol permits identification of PINA and PINB proteins that are bound to the surface of starch granules and also unbound. Values are relative levels TX114-extractable PIN proteins in transgenic lines versus levels in Heron, with SE mean in parentheses

[c] The friabilin protocol identifies only that fraction of PINA and PINB proteins that are bound to the surface of starch granules. Values are relative levels of starch granule-bound PIN proteins in transgenic lines versus Heron, with SE mean in parentheses

transgenically expressed wild type *Pina* and *Pinb* sequences in a hard genotype with mutated, but perhaps partially functioning *Pinb*. Soft grain texture obtained when *Pinb* was added compared to intermediate grain texture when *Pina* was added raises questions about whether the differing results are because *Pina* was present without a fully functional *Pinb*, or because *Pina* is merely less functional than *Pinb*. The present study addresses the effects of adding *Pina* where no native *Pina* is present, and circumvents limitations of the previous transgenic studies.

Expression of the *Pina* sequence resulted in softer grain compared to non-transformed Bobwhite (Table 1). The three transgenic lines were not different from each other for grain texture. The soft grain phenotype was accompanied by increased but varying amounts of *Pina* transcript accumulation (Fig. 2), total PINA, and friabilin and its components: starch bound PINA and PINB (Table 2). In all instances these amounts were higher than the soft wheat reference sample (Table 2). Capparelli et al. (2003) concluded *Pina* expression was involved in regulating starch bound friabilin in a set of hard and soft cultivars. Our transgenic lines provide an isogenic comparison of varying *Pina* expression and friabilin. Although we did not absolutely quantify *Pina* expression, our results show increased friabilin occurred with the expression of *Pina* in the three transgenic lines.

Hogg et al. (2004) characterized starch bound PINA and PINB and showed no increase in starch bound PINB with addition of *Pina* while PINA and PINB increased with addition of *Pinb* alone or both *Pina* and *Pinb*. This led Hogg et al. (2004) to conclude that both PINA and PINB are needed to form friabilin in order to obtain soft grain. Swan et al. (2006) crossed Hi-Line transgenic lines expressing *Pina* or *Pinb* to a soft wheat and found progeny with *Pinb* had softer grain than those with *Pina*. They concluded PINB might be the limiting factor in friabilin formation and subsequent grain softness in soft wheat. The untransformed Bobwhite showed no starch bound PINA or PINB. That result agrees with Capparelli et al. (2003) who found *Pina-D1b* (*Pina* null allele) genotypes had no PIN bound to starch. Our results showed addition of *Pina* gave increased, though varying, amounts of starch bound PINA and PINB in the three transgenic lines. A plausible explanation might be that since both PINA and PINB are needed for friabilin formation, high amounts of PINA brought about by over expression of *Pina* may dilute or out compete PINB if starch granule surface binding sites are limited. The line with lowest amount of PINA (BW3) has highest amount of PINB on starch, and the line with highest amount of PINA (BW2) has lowest amount of starch bond PINB. Our results seem to support the conclusion that relative amounts of PINA and PINB are more important for friabilin formation than total PIN. Grain texture phenotype was similar for the three transgenic lines despite varying amounts of total and starch bound PIN. Hogg et al. (2004) found different events for the addition of *Pina* and *Pinb* gave different total PIN and starch bound PIN, and soft grain where SKCS hardness was not different. Phenotypic differences may have been detected among the three transgenic lines had other indicators of grain texture such as particle size index been measured.

Beecher et al. (2002) showed the soft phenotype was restored by complementing the mutated *Pinb-D1b* allele in Hi-Line with the wild type *Pinb-D1a* sequence. Hogg et al. (2004) investigated the effects of transgenicly adding *Pina*, *Pinb*, or both *Pina* and *Pinb* in the same Hi-Line background. Softest grain resulted

from addition of *Pinb* alone or both *Pina* and *Pinb*, with addition of *Pina* alone giving intermediate grain texture. Our results coupled with Hogg et al. (2004) demonstrate that the effect of added *Pina* depends on the native *Pin* genotype. Giroux and Morris (1997 and 1998) postulated that the hard phenotype results from sequence alteration in either *Pina* or *Pinb* that affects protein expression or function. The present study, Beecher et al. (2002) and Hogg et al. (2004) further support this hypothesis by showing the soft phenotype can be restored by complementing either mutated *Pinb* or null *Pina* allele with the corresponding wild type *Pin* allele.

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