Effect of Multiple Copies of Puroindoline Genes on Grain Softness

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ABSTRACT

End use quality in wheat (*Triticum aestivum* L.) is primarily determined by grain hardness or texture. The puroindoline genes *Pina* and *Pinb* are the main components of the 15-kDa friabilin protein, which is associated with kernel softness. *Pina* and *Pinb* are expressed in diploid wheat species but are silent in tetraploid wheat. The active puroindolines in hexaploid, or bread wheat, were derived from *Aegilops tauschii* Coss., the D genome diploid donor. The focus of this study was to incorporate active puroindoline genes into the A and B genomes of bread wheat and analyze their impact upon grain softness. Functional copies of *Pina* and *Pinb* in disomic substitution lines of *T. monococcum* L. chromosome $5A^m$ for 5A of *T. aestivum* and $5S^s$ of *Aegilops searsii* Feldman & Kislev ex K. Hammer for 5B of *T. aestivum* were used to produce lines that contained four copies ($5A^m$, 5D; $5S^s$, 5D) and six copies ($5A^m$, $5S^s$, 5D) of the puroindolines. There was a direct correlation in grain softness with the increase in copy number of the puroindolines. Northern blots showed increased expression of both *Pina* and *Pinb*. Extraction of TX114 soluble proteins indicated that levels of both proteins were also increased. Single kernel characterization system (SKCS) analysis showed a decrease in kernel hardness by approximately 10 points below the value of 71 for 'Chinese Spring' (CS) for each additional copy of *Pina* and *Pinb* added. These results indicate that increasing the functional copy number of the puroindolines can impact grain softness in bread wheat.

WHEAT IS A STAPLE FOOD for half of the world's population. One of the defining characteristics for milling and baking of wheat is the kernel texture. The manifestation of kernel texture, or grain hardness in end use quality, results in reduced particle size and finer-textured flour in soft wheat used for cookies, cakes, and pastries, and larger, coarser-textured flour in hard wheat used for bread (Morris and Rose, 1996). Bread wheat, *T. aestivum*, has both soft and hard cultivars. Durum wheat, *T. turgidum* L., which is used to produce pasta, has an extremely hard texture.

Differences in kernel texture were first characterized by Cobb (1896), and later more precise instruments in measuring grain texture were used to determine the inheritance of grain hardness (Symes, 1965; Mattern et al., 1973). A single locus *Ha* was identified on the short arm of chromosome 5D for grain hardness (Law et al., 1978). Greenwell and Schofield (1986) observed a positive association between a 15-kDa starch protein termed friabilin and grain softness. All soft wheat had a prominent 15-kDa band, the hard wheat had a faint band, and the durum wheat lacked this band completely. Amino acid sequencing of the friabilin protein linked grain hardness to the puroindoline genes (Blochet et al., 1991, 1993). Igrejas et al. (2001) studied the impact of environmental effects on puroindoline content and concluded that both the puroindolines and grain hardness were not significantly affected by different growing conditions.

Nitrogen-terminal sequencing of friabilin indicated two proteins (Oda and Schofield, 1997). The two proteins are the puroindoline proteins *Pina* and *Pinb* (Jolly et al., 1993; Morris et al., 1994). *Pina* and *Pinb* are 55% similar at the cDNA level and contain a unique tryptophan-rich hydrophobic domain (Gautier et al., 1994). The expression of both *Pina* and *Pinb* are needed for soft kernel texture. A null mutation within the *Pina* sequence or point mutations and null mutations within the *Pinb* sequence are associated with hard wheat kernel texture (Giroux and Morris, 1997, 1998). Predominantly point mutations within the *Pinb* loci account for hard wheats, and have been complemented by transformation with the soft-type *Pinb* sequence (Beecher et al., 2002). There are nine different alleles known at the puroindoline locus. In *Pina* there are two alleles, *Pina-D1a* (soft, wild-type) and *Pina-D1b* (null hard). In the *Pinb* locus there are seven alleles, *Pinb-D1a* (soft wild-type) and six hard alleles *Pinb-D1b* through *Pinb-D1g* (Morris, 2002). In *T. aestivum,* both *Pina* and *Pinb* have been mapped to the distal region on chromosome arm 5DS (Sourdille et al., 1996; Giroux and Morris, 1997). In diploid wheat, both *Pina* and *Pinb* and *Gsp-1a*, another protein associated with softness, are contained within a single 105-kb bacterial artificial chromosome (Tranquilli et al., 1999). The puroindoline genes are present in the diploid progenitor species, but both *Pina* and *Pinb* were lost during the evolution of tetraploid wheat (Gautier et al., 2000). The aim of this study was to introduce functional copies of the puroindoline genes into the A and B genomes of bread wheat, and to study the effects of multiple copies of the puroindoline genes on kernel hardness, friabilin levels, and expression of three copies of puroindoline genes in a polyploid genome.

MATERIALS AND METHODS

Disomic substitution lines TA6642 $DS5A^m$(5A) and TA6562 $DS5S^s$(5B) were used to incorporate functional copies of *Pina* and *Pinb* into the hexaploid wheat CS. In $DS5A^m$(5A), null allele on chromosome 5A of CS is substituted by a functional copy from $5A^m$ of *T. monococcum* (Kota and Dvorak, 1998, unpublished data). In $DS5S^s$(5B), null copy on 5B of CS is

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Abbreviations: CS, Chinese Spring; RFLP, restriction fragment length polymorphism; SDS, sodium dodecyl sulfate; SKCS, single kernel characterization system; TBE, tris-borate-ethylenediaminetetraacetic acid.

substituted by functional copies from S^s *A. searsii* (Friebe et al., 1995). The disomic substitution lines are maintained by the Wheat Genetics Resource Center, Kansas State University. DS5A^m(5A) and DS5S^s(5B) were crossed, and F_1 plants were selfed, all subsequent filial generations were derived by single-seed descent. Microsatellite screening was performed on F_2 plants. Restriction fragment length polymorphism analysis (RFLP), friabilin, and SKCS test were performed on F_3 plants. Northern analysis and Triton X-114 soluble protein tests were performed on seeds from F_4 plants. The plants for the SKCS and friabilin assays were grown in Bozeman, MT. The F_3 plants for RFLP and the F_4 plants for northern analysis were grown in the greenhouse at Kansas State University.

DNA was isolated from F_2 plants following the protocol of Faris et al. (2000). Microsatellite markers *gwm415* and *gwm159* which map on the short arm of both chromosome 5A and 5B, respectively, were used to screen 384 F_2 plants for segregants incorporating 5A^m for CS 5A and 5S^s for CS 5B chromosomes. Polymerase chain reaction conditions and annealing temperatures followed the guidelines of Roder et al. (1998). Marker segregation was determined on polyacrylamide gels, (6.0% acrylamide; 19:1 acrylamide-to-bisacrylamide ratio), in a 0.5 × tris-borate-ethylenediaminetetraacetic acid (TBE) buffer. Electrophoresis was run at 200 V for 3 h (See et al., 2000).

Filter hybridization, probe labeling, and filter washing were conducted following the protocol of Faris et al. (2000). Specifically, 20 μg of DNA digested with *Hin*dIII was electrophoresed in a 0.8% w/v, 1 × TBE agarose gel at 22 V for 24 h. Prehybridization was done in 50 mL of 5 × Denhardt's solution (0.1% Ficoll; 1 mg mL^{-1} *N,O*-Bis(trimethylsilyl)acetamide; 1 mg mL^{-1} polyvinylpyrrolidone), 6 × SSPE (0.9 *M* NaCl, 0.6 *M* NaH_2PO_4), 0.05 mg mL^{-1} denatured salmon sperm DNA, and 0.5% sodium dodecyl sulfate (SDS). After incubation at 65°C for 16 h, prehybridization solution was replaced with 4 mL of hybridization solution consisting of 5 × Denhardt's solution, 6 × SSPE, 0.5% SDS, 0.05 mg mL^{-1} denatured salmon sperm, and 20% dextran sulfate. Probes were amplified from CS with the primers for *Pina* and *Pinb* following procedures as described in Gautier et al. (1994).

Northern-blot analysis was conducted following the protocol of Giroux and Morris (1997). RNA was isolated from developing wheat seeds at 20 d post anthesis by a LiCl method (McCarty, 1986). Two micrograms of RNA was loaded on a formaldehyde agarose gel, then blotted to a nylon membrane and hybridized with *Pina* and *Pinb* probes following the same hybridization procedure as previously described. Sample loading was standardized with rRNA. Sample variation was normalized by reprobing the *Pina* and *Pinb* blots with a *glutenin* gene probe pGlu10H5 described by Blechl and Anderson (1996). Gene expression levels were used to produce adjusted normalized *Pina* and *Pinb* expression levels. RNA from CS was loaded in increasing concentrations from 0.5× to 4× as a guideline for increased expression levels. Grain hardness was measured with Model SKCS 4100 (Perten Instruments, Springfield, IL). F_3 plants were grown in a field in Bozeman, MT, in 2001. When seed numbers permitted, 100 seeds were used per replication, and three replications were used. The mean of the 100 seeds was subjected to statistical analysis for grain hardness compared against CS with the SAS mixed procedure (SAS Institute, 1998).

Friabilin was isolated from 60 mg whole wheat flour as previously described (Bettge et al., 1995). TX114 protein extracts were prepared from whole meal flour as described previously (Giroux and Morris, 1998). After dilution to the optimal concentration for visualization, 10-μL aliquots were separated in 10 to 20% w/v tris-glycine gradient gels at 130 V and stained with Coomassie blue (Sambrook et al., 1989).

RESULTS

The initial screening of the 384 F_2 plants from the cross DS5A^m(5A) × DS5S^s(5B) was performed with microsatellite *gwm415* marker for 5A, which showed a size polymorphism between CS and DS5A^m(5A) in the 5A^m lines. Eighty-four F_2 plants, approximately 1/4 of the total were homozygous for 5A^m chromosome. The 84 F_2 plants containing the *gwm415* diagnostic fragment were then screened with *gwm159* marker for 5B/5S^s for the presence of the 5S^s diagnostic band; 25 of the 384 F_2 plants contained both diagnostic fragments for 5A^m and 5S^s. This number was close to the 1/16 expected ratio for incorporation of both chromosomes. To further verify microsatellite marker results, a subset of the 84 F_2 plants were selected for assaying the *Pina* and *Pinb* loci by RFLP analysis. Table 1 indicates the genotypes of the F_3 and F_4 lines used in this study.

Southern analysis of *Hin*dIII digested genomic DNA with *Pina* and *Pinb* probes detected one band in each case on 5D chromosome of CS, and null alleles at 5A and 5B. An additional band of different molecular weight was observed in DS5A^m or DS5S^s lines (Fig. 1, *Pina*, *Pinb*). In the disomic substitution lines, the null alleles at 5A and 5B have been substituted by the puroindoline a and b loci derived from the 5A^m and 5S^s chromosomes of *T. monococcum* and *A. searsii*, respectively. Three fragments were detected in lines 12, 30, and 284, corresponding to the puroindoline loci on 5D, 5A^m, and 5S^s chromosomes. Lines 4 and 196 had less intensity at one band due to less incorporation of ^{32}P probe in the 5A^m fragment, indicating that the 5A locus was heterozygous. Restriction fragment length polymorphism analysis of the F_4 plants confirmed that the 5A^m chromosome was heterozygous in the F_3, and was not recovered in the next generation (data not shown).

Table 2 shows the SKCS results for CS and the F_3 lines containing two to six copies of puroindoline genes. A hardness value of 71 was obtained for CS with two copies at the 5D locus, which was used as a baseline reference. All lines containing from four to six copies at the puroindoline locus showed decreased hardness ranging from 46 for line 12 (six copies) to 60 for line 127 (four copies); compared with CS line 127 with four

Table 1. Chromosome constitution and *Pina* and *Pinb* copy number in 'Chinese Spring' (CS) and F_3 and F_4 wheat lines from the cross DS5A^m(5A)/DS5S^s(5B).

Plant ID†	Copies	F_3 Genotype	F_4 Genotype
127	4	18″ + 5A″ + 5S$^{s''}$ + 5D″	18″ + 5A″ + 5S$^{s''}$ + 5D″
4, 196	5	18″ + 5A$^{m'}$/5A′ + 5S$^{s''}$ + 5D″	18″ + 5A″ + 5S$^{s''}$ + 5D″
6, 12, 30, 284	6	18″ + 5A$^{m''}$ + 5S$^{s''}$ + 5D″	18″ + 5A$^{m''}$ + 5S$^{s''}$ + 5D″

† CS genotype is 18″ + 5A″ + 5B″ + 5D″.

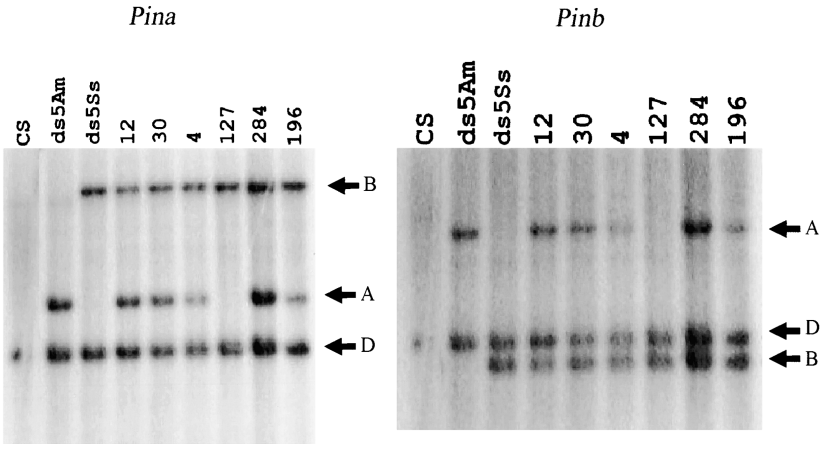

Fig. 1. ***Pina*** **and** ***Pinb*** **gene loci number in different lines at the F_3 generation determined by restriction fragment length polymorphism analysis. 'Chinese Spring' (CS) is homozygous at the 5D locus and has two copies. Other lines have four or six copies except lines 4 and 196, which have five copies each as they were heterozygous at the 5A locus as indicated by reduced ^{32}P signal and their segregation in the F_4 generation (data not shown).**

copies, hardness decreased in value by approximately 10 points. Lines 4 and 196, which upon later analysis proved to be heterozygous at the $5A^m$ loci and thus had five copies, showed a lower value than line 127 with four copies (Table 2). Lines 12, 30, and 284, each with six copies, all had hardness values approximately 20 points lower than CS. The SKCS data indicate that increasing copies of the puroindoline genes generally decreases hardness.

Increasing the copy number of puroindoline genes by incorporating loci at $5A^m$ and $5S^s$ chromosomes showed increased expression of the puroindoline genes present in seeds collected at 20-d post anthesis (Fig. 2). Expression analysis showing *Pina*, adjusted *Pina*, *Pinb*, and adjusted *Pinb* represents the original and normalized levels of expression based upon normalization with *glutenin* gene expression levels. *Pina* expression was increased in all progenies over that of CS and the parental lines (Fig. 2). The adjusted values of *Pina* showed a range from 1.5 times to 4 times greater than CS expression compared with the incremental loaded samples of CS used as a baseline reference. Loci copy number is, however, not directly correlated at the mRNA level. Line 4 with four copies and lines 12 and 284 with six copies had *Pina* expression levels lower than the copy number indicated by Southern analysis and what would be predicted by SKCS data. Lines 12 and 284 with six copies had expression levels less than two times greater than CS. Line 4 with four copies had an expression level only 1.5 times greater than CS. Lines 127 and 196 with four copies had expression levels that correlated with

Table 2. Hardness of F_3 lines with increasing ***Pina*** **and** ***Pinb*** **copy number based upon a single kernel characterization system test. 'Chinese Spring' (CS) was used as a baseline for statistical significance for increased hardness.**

	Hardness†						
	71.47	59.54	49.80	53.95	50.73	47.20	46.60
	Plant ID‡						
Plant ID	CS(2)	127(4)	4(5)	196(5)	30(6)	284(6)	12(6)
CS	–						
127	**	–					
4	***	**	–				
196	***	§	ns¶	–			
30	***	*	ns	ns	–		
284	***	*	ns	ns	ns	–	
12	***	***	ns	*	ns	ns	–

* Significant at the 0.05 level of probability.
** Significant at the 0.01 level of probability.
*** Significant at the 0.001 level of probability.
† Numbers of replications: (100 seeds per replication). CS, 127, 4, and 12 were replicated four times. Lines 196, 30, and 284 had 3, 2, and 1 replications, respectively.
‡ The numbers in parentheses indicate puroindoline loci copy number.
§ Significant at the 0.1 level of probability.
¶ ns = not significant at the levels tested.

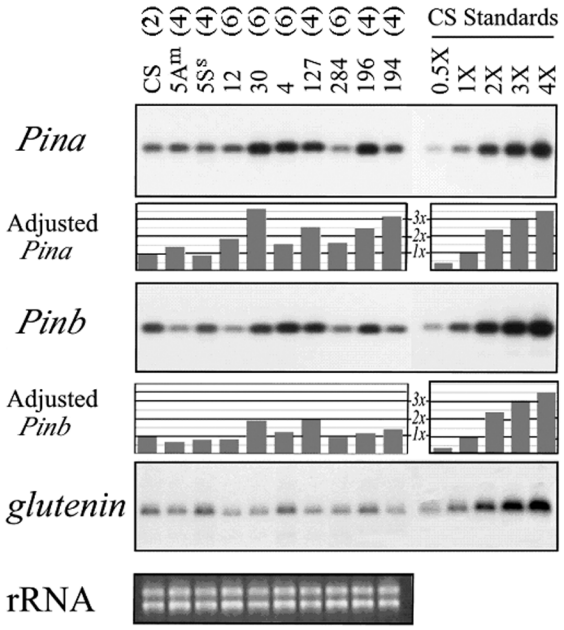

Fig. 2. Puroindoline expression analysis. RNA was extracted from 20-d post anthesis seeds. Membranes were probed with ***Pina*** **and** ***Pinb*****. Glutenin levels were used to normalize loading differences. 'Chinese Spring' (CS) was loaded in concentrations from 0.5× (one copy) to 4× (eight copies) as a reference. The adjusted values indicate a graphical representation of the expression levels compared with CS standards. The numbers in parentheses indicate puroindoline copy number.**

copy number based upon the CS standards and what would be expected based upon RFLP puroindoline loci copy number. Line 30 with six copies was the only line that showed an expression level higher than would be expected with an expression level four times greater than CS. The expression results for the *Pinb* levels were more variable and expression levels were lower than in *Pina* in all lines (Fig. 2). The majority of the lines had expression levels which were near the same level as CS regardless of puroindoline copy number; this included four-copy lines 4 and 196, and six-copy lines 12 and 284. Line 127 with four copies was the only line with the expected expression level in both the *Pina* and *Pinb* analysis. Line 30, which had four-fold greater expression level in *Pina*, showed only a two-fold increase in expression level in *Pinb*. Line 4, while below its two-fold expected level, showed the most consistency between *Pina* and *Pinb* expression assays.

It was next determined if the increased level of the *Pina* and *Pinb* RNA correlates with increased production of the puroindoline proteins bound to the surface of starch as friabilin and extractable with TX114 (Fig. 3). Chinese Spring was included as a baseline for friabilin concentrations with two copies of puroindoline genes. The tetraploid durum wheat cultivar Langdon was included to show lack of friabilin as it has null alleles on the 5A and 5B chromosomes. A Langdon 5D(5B) substitution line was also used to indicate the reintroduction of the friabilin protein in a tetraploid wheat by a 5D chromosome with functional copies of the puroindolines. All the lines tested had higher concentrations of friabilin than CS, demonstrating that the extra puroindoline gene copies correspond to increased friabilin levels. Lines 6 and 12, having six copies, show a definite increase in friabilin protein. Lines 4 and 127, with four puroindoline genes, although loaded at less concentration than CS, still show an increase in friabilin level over the baseline level indicated by CS and the Langdon 5D(5B) substitution line.

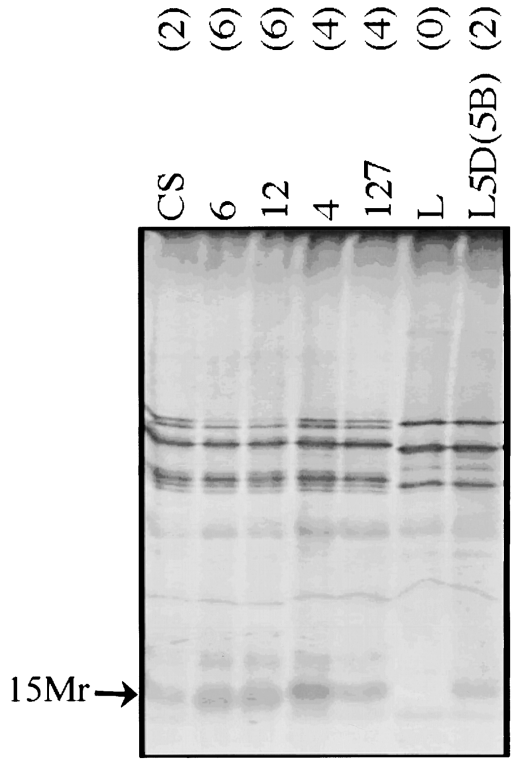

Fig. 3. Starch surface protein levels, though not normalized, show a clear increase in starch surface protein levels that can be seen in lines 6, 12, 4, and 127, as indicated by the 15Mr (relative mobility) fragment. Starch surface protein is absent in the durum cultivar Langdon (L), but reappears at similar levels to 'Chinese Spring' (CS) in the Langdon 5D(5B) substitution line L5D(5B) that contains the 5D chromosome of CS. The numbers in parentheses indicate puroindoline copy number.

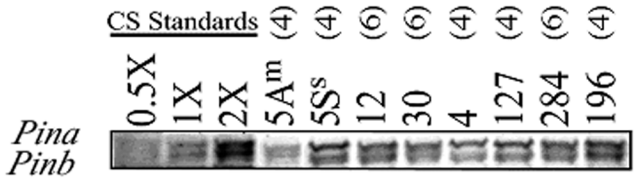

Fig. 4. Friabilin proteins from 'Chinese Spring' (CS) and the lines with multiple copies of *Pina* and *Pinb* genes. The friabilin levels indicated by the upper *Pina* and lower *Pinb* bands show an increased concentration of friabilin in the lines 12, 30, and 284, each with three copies of the puroindoline genes. Lines 4 and 127 with two copies produce less protein, but are slightly more concentrated than the 1× (two copy) CS standard. Line 196 with two copies shows increased friabilin levels above that of other two-copy lines. The numbers in parentheses indicate puroindoline copy number.

Isolated starch surface proteins show an increased concentration of protein in lines 12, 30, and 284, each with six copies of the puroindoline genes (Fig. 4). Lines 4 and 127 with four copies produce less protein, but are slightly more concentrated than the 1× CS standard. Line 196 with four copies had increased protein level above that of other four-copy lines. In the lines tested, it is interesting to note that the upper *Pina* band had an increased concentration over the lower *Pinb* band. These data, combined with northern analysis and SKCS data, indicate that additional copies of the puroindoline genes in hexaploid wheat increase the amount of *Pina* and *Pinb* expression and also increase friabilin and starch surface protein levels, with an end result being decreased kernel hardness.

DISCUSSION

Wheat kernel texture is one of the major determining factors that dictates end use quality. The tetraploid progenitor of modern cultivated hexaploid wheat is extremely hard, which may be because of the absence of expression of the hardness locus (Gautier et al., 2000). This is because of the elimination of genes from chromosomes 5A and 5B. Hexaploid wheat acquired a functional copy of the hardness loci from its hybridization with *A. tauchii* to *T. turgidum* (McFadden and Sears, 1946). In this study, functional copies of the puroindoline genes were reintroduced into bread wheat, and grain hardness was evaluated. The SKCS data showed that multiple copies of *Pina* and *Pinb* reduced hardness in the kernels. Each additional copy reduced hardness by approximately 10 points below that of CS. This was also observed previously by Tranquilli et al. (2002) in a 5A/5A^m substitution line. However, there was not a

linear correlation between some samples and their indicated copy number for SKCS hardness data. Lines 4 and 196, which were heterozygous ($5A^m$/5A) with five copies of puroindoline genes, had hardness values as low as six-copy lines such as 30 and 284. Even in a heterozygous state, the addition of the $5A^m$ locus had a large impact on hardness value.

In the expression assays, for most of the lines, expression increased with additional copies of puroindoline genes. *Pina* expression more closely mirrored the copy number indicated by RFLP, although lines 12 and 284 showed values less than expected. *Pinb* expression was below the levels of *Pina* for all lines tested. Most lines did not show an increase in expression above the baseline expression level seen in CS. Line 127 was the only normal line in this aspect. Gautier et al. (1994) observed a higher level of *Pina* transcripts over that of *Pinb* transcripts from developing seeds 4 to 44 d after flowering. Overall, lines 127 and 30 had the most consistent results in kernel hardness and expression assays. The friabilin levels in the four- and six-copy lines indicate that increased *Pina* and *Pinb* expression is converted into increased friabilin levels. The friabilin protein consists primarily of a 1:1 ratio of *Pina* and *Pinb* (Jolly et al., 1993; Morris et al., 1994). Our data indicate that this ratio is not maintained when multiple copies of *Pina* and *Pinb* genes are introduced in hexaploid wheat. It is evident that regulation and expression of the multiple copies of puroindoline genes in a polyploid genome is not straightforward. This regulation appears to retard the expression of the *Pinb* gene more severely than the *Pina* gene, with *Pina* showing expected values in four of the lines tested. Although expression appeared to be suppressed, the expected increased softness in kernel texture was observed. Further analysis into the individual expression of the puroindoline genes from each of the genomes would help to elucidate the possible mechanisms behind this phenomenon.

Beecher et al. (2002) showed through transformation that the addition of the *Pinb-D1a* wild-type soft gene into a hard wheat line 'Hi-Line' reduced hardness. This evidence indicates two things, first that introduction of a soft *Pinb* gene in a hard background can affect grain hardness. More importantly, it showed a direct role of puroindolines on grain hardness. A similar experiment by Tranquilli et al. (2002) indicated this as well by showing that a deletion in the short arm of 5DS-2 which encompasses the puroindoline alleles, drastically increased hardness values above that of euploid CS, while deletions in the other group 5 chromosomes did not have an effect on grain hardness. Our results also demonstrate the importance the puroindoline genes in determining grain hardness. Northern analysis and protein levels indicate increased RNA and friabilin levels. The SKCS data showed that the incorporation of the puroindoline loci from $5A^m$ and $5S^s$ decreased kernel hardness by 10 points for each addition copy incorporated into hexaploid wheat.

The incorporation of the $5A^m$ and $5S^s$ chromosomes containing functional copies of the puroindoline genes into bread wheat demonstrated that additional copies of the hardness loci increased kernel softness. To develop useful soft wheat lines, these traits must be transferred into agronomically elite soft lines and end use milling and baking quality evaluated. However, the soft lines represented here are not suitable for such purposes. In these lines, $5S^s$ is substituting for 5B and they are lacking the *Ph1* gene which controls diploid-like meiosis of hexaploid wheat (Riley and Chapman, 1958) and will be cytolotogically unstable. The next step in the development of this germplasm will be to obtain a translocation line where the short arm of chromosome $5S^s$ is translocated to the long arm of 5B, carrying the *Ph1* gene.

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