Effect of Multiple Copies of Puroindoline Genes on Grain Softness

D. R. See, M. Giroux, and B. S. Gill*

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* for 5A of T. aestivum and 5S* of Aegilops searsii Feldman & Kislev ex K. Hammer for 5B of T. aestivum were used to produce lines that contained four copies (5A*, 5D; 5S*, 5D) and six copies (5A*, 5S*, 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. Amino acid sequencing of the friabilin protein linked grain hardness to the puroindoline genes (Blochet et al., 1999). The puroindoline genes are present in all wheat species 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 DSS5a(5A) and TA6562 DSS5s(5B) were used to incorporate functional copies of Pina and Pinb into the hexaploid wheat CS. In DSS5a(5A), null allele on chromosome 5A of CS is substituted by a functional copy from 5A* of T. monococcum (Kota and Dvorak, 1998, unpublished data). In DSS5s(5B), null copy on 5B of CS is

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* A. searsii (Friebe et al., 1995). The disomic substitution lines are maintained by the Wheat Genetics Resource Center, Kansas State University. DSS5*(5A) and DSS5*(5B) were crossed, and F1 plants were selfed, all subsequent filial generations were derived by single-seed descent. Microsatellite screening was performed on F1 plants. Restriction fragment length polymorphism analysis (RFLP), friabilin, and SKCS test were performed on F1 plants. Northern analysis and Triton X-114 soluble protein tests were performed on seeds from F2 plants. The plants for the SKCS Northern analysis and Triton X-114 soluble protein tests were stained with Coomassie blue (Sambrook et al., 1989). Selfed, all subsequent filial generations were derived by single-tract crosses. Prehybridization was done in 50 mL of 5 × Denhardt’s solution (0.1% Ficoll; 1 mg mL⁻¹ N,N-Bis(trimethylsilyl)acetamide; 1 mg mL⁻¹ polyvinylpyrrolidone), 6 × SSPE (0.9 M NaCl, 0.6 M NaH₂PO₄), 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 seed 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). F1 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 (Betige 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 F2 plants from the cross DSS5*(5A) × DSS5*(5B) was performed with microsatellite gwm415 marker for 5A, which showed a size polymorphism between CS and DSS5*(5A) in the 5Am lines. Eighty-four F2 plants, approximately 1/4 of the total were homozygous for 5Am chromosome. The 84 F2 plants containing the gwm415 diagnostic fragment were then screened with gwm159 marker for 5B/5S for the presence of the 5S diagnostic band; 25 of the 384 F2 plants contained both diagnostic fragments for 5Am and 5S. 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 F2 plants were selected for assaying the Pina and Pinb loci by RFLP analysis. Table 1 indicates the genotypes of the F1 and F2 lines used in this study.

Southern analysis of HindIII-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 DSS5*(5A) or DSS5*(5B) 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 5Am and 5S 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, 5Am, and 5S chromosomes. Lines 4 and 196 had less intensity at one band due to less incorporation of 32P probe in the 5Am fragment, indicating that the 5A locus was heterozygous. Restriction fragment length polymorphism analysis of the F2 plants confirmed that the 5Am chromosome was heterozygous in the F1, and was not recovered in the next generation (data not shown).

Table 1 shows the SKCS results for CS and the F1 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>
<thead>
<tr>
<th>Plant ID1</th>
<th>Copies</th>
<th>F1 Genotype</th>
<th>F1 Genotype</th>
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<tbody>
<tr>
<td>127</td>
<td>4</td>
<td>18° + 5A° + 5S° + 5D°</td>
<td>18° + 5A° + 5S° + 5D°</td>
</tr>
<tr>
<td>4, 196</td>
<td>5</td>
<td>18° + 5A°/5A + 5S° + 5D°</td>
<td>18° + 5A° + 5S° + 5D°</td>
</tr>
<tr>
<td>6, 12, 30, 284</td>
<td>6</td>
<td>18° + 5A° + 5S° + 5D°</td>
<td>18° + 5A° + 5S° + 5D°</td>
</tr>
</tbody>
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† CS genotype is 18° + 5A° + 5B° + 5D°.
copies, hardness decreased in value by approximately 10 points. Lines 4 and 196, which upon later analysis proved to be heterozygous at the 5A<sup>a</sup> locus 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<sup>a</sup> and 5S<sup>s</sup> 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 copies 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

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**Table 2. Hardness of F<sub>3</sub> lines with increasing Pina and Pinb copy number based upon a single kernel characterization system test.**

<table>
<thead>
<tr>
<th>Plant ID</th>
<th>127(4)</th>
<th>30(6)</th>
<th>284(6)</th>
<th>12(6)</th>
</tr>
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<tr>
<td>Hardness†</td>
<td>71.47</td>
<td>59.54</td>
<td>49.80</td>
<td>53.95</td>
</tr>
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</table>

**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.

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* 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 12, 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.
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.

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× (two copy) 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/5Aa 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<sup>a</sup>/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<sup>a</sup> 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* expression (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 individial 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<sup>a</sup> and 5S<sup>s</sup> decreased kernel hardness by 10 points for each addition copy incorporated into hexaploid wheat.

The incorporation of the 5A<sup>a</sup> and 5S<sup>s</sup> 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 agriculturally 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<sup>s</sup> 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 cytologically unstable. The next step in the development of this germplasm will be to obtain a translocation line where the short arm of chromosome 5S<sup>s</sup> is translocated to the long arm of 5B, carrying the *Ph1* gene.

REFERENCES


