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# Substitutions and Deletions of Genes Related to Grain Hardness in Wheat and Their Effect on Grain Texture

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## ABSTRACT

**The *Hardness* (*Ha*) locus on chromosome 5D is the main determinant of grain texture in hexaploid wheat (*Triticum aestivum* L.). Puroindoline (*Pina-D1*, *Pinb-D1*) and Grain Softness Protein (*Gsp-D1*) genes are tightly linked at this locus. Additional copies of the *Gsp-1* gene are present on chromosomes 5A and 5B. Mutations in the *Pina-D1* and *Pinb-D1* genes have been individually associated with grain hardness, but it is not known if mutations at both loci may further increase hardness or if additional copies may reduce it. In addition, there is no clear evidence of the effect of the *Gsp-1* genes on grain texture. To answer these questions, we compared the effect of different dosages of puroindoline and *Gsp-1* genes on grain texture. Isogenic substitution and deletion lines for homoeologous group 5 in 'Chinese Spring' (CS) were evaluated in two replicated field trials with 13 blocks each. Deletions or allelic variants of *Gsp-A1* and *Gsp-B1* did not produce significant effects on grain texture, suggesting that these genes do not have a critical role in grain hardness. Simultaneous deletions of *Pina-D1* and *Pinb-D1* in deletion line 5DS-2 and substitution line CS (Red Egyptian 5D) resulted in significantly higher hardness index values than all other lines including CS (Timstein 5D) carrying a single *Pina-D1* deletion ($P = 0.02$). The incorporation of additional copies of *Pina-A$^m$1* and *Pinb-A$^m$1* from *T. monococcum* L. in recombinant substitution line 5A/5A$^m$ in CS resulted in significantly softer grains than those from the CS control ($P < 0.01$).**


Wheat marketing systems established a primary classification of hexaploid wheat based on endosperm texture, that is, the hardness or softness of the grain. This trait determines many of wheat's potential end-uses. Hard textured grains require more grinding energy than soft textured grains to reduce endosperm into flour, and during this milling process a larger number of starch granules become physically damaged. Since damaged starch granules absorb more water than undamaged granules, flours from hard wheats are preferred for yeast-leavened bread baking, while flours from soft wheats are preferred for manufacturing cookies and cakes (Tippless et al., 1994).

Different approaches have been used to characterize the natural variation observed in this character. Genetic analyses have shown that endosperm texture is primary controlled by the *Hardness* (*Ha*) locus on the short arm of chromosome 5D (Mattern et al., 1973; Law et al., 1978). It is a simply inherited character (Symes, 1965), and although the main locus is referred as *hardness*, softness is in fact the dominant trait.

Biochemical studies suggested that differences exist in the binding strength of starch granules with the surrounding protein matrix between soft and hard wheat varieties (Pomeranz and Williams, 1990). A starch surface-associated protein ($M_r$ 15kd) was found at high levels in soft wheat and at relatively low levels in hard wheat (Greenwell and Schofield, 1986). This protein, referred to as *friabilin*, became a putative marker for softness. The accumulation of friabilin in seeds was found to be controlled by the short arm of chromosome 5D, reinforcing the idea that friabilin could be the product of the *Ha* locus (Jolly et al., 1993).

Friabilin was found to be a composite of related lipid-binding proteins including the basic cysteine–rich proteins puroindolines a (PINA) and b (PINB) and the grain softness protein family GSP-1 including GSP-1a, GSP-1b and GSP-1c (Gautier et al, 1994; Rahman et al., 1994). The main components have been shown to be the puroindolines a and b. Molecular studies revealed that mutations in the puroindoline gene sequences were present in all hard-textured wheats. Either a deletion resulting in the complete lack of PINA protein, or single -nucleotide mutations resulting in a modified amino acid sequence or null expression of the PINB protein were shown to be inseparably linked to hard-textured grains in surveys of American and European wheats (Giroux and Morris, 1997; 1998; Lillemo and Morris, 2000; Morris et al., 2001). These results suggest a direct role for the effect of the puroindolines on grain texture. No clear evidence on the role of *Gsp-1* genes on grain texture has been obtained so far.

Genes encoding these proteins have been mapped on the distal part of chromosome 5DS completely linked to the *Ha* locus. Puroindoline loci, *Pina-D1* and *Pinb-D1*, have been detected only on chromosome 5D, while homoeologous *Gsp-1* loci have been conserved in all the three genomes, on chromosomes 5A, 5B and 5D (Dubcovsky and Dvorak, 1995; Jolly et al., 1996; Sourdille et al., 1996; Giroux and Morris, 1997). Puroindoline genes from chromosomes 5A and 5B are present in the diploid donors of the A and B genomes but have been deleted in polyploid wheats (Tranquilli et al., 1999; Gautier et al., 2000). The *Ha*-related genes may act together to affect grain softness, and it was observed that genotypes having the deletion at *Pina-D1* were harder than those having the single nucleotide mutation in *Pinb-D1*

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**Abbreviations:** ANCOVA, analysis of covariance; ANOVA, analysis of variance; CS, 'Chinese Spring'; G × E, genotype × environment; GSP, grain softness protein; PINA, PINB, puroindoline a and b proteins; RCBD, randomized complete block design; RFLP, restriction fragment length polymorphism; SKCS, single kernel characterization system.

(Giroux et al., 2000). In consequence, it is possible to speculate that the current range of grain textures available in hexaploid wheat could be expanded to harder textures if deletions for both puroindoline genes were combined in one genotype or to softer textures if active genes from the diploid species were introduced. The main objective of our study was to test these two hypotheses. An additional objective was to test the effect of the *Gsp-A1* and *Gsp-B1* loci on grain texture.

## MATERIALS AND METHODS

### Plant Material

Different lines developed in the isogenic background of CS were selected for this evaluation. They included: (i) Deletion lines CS Del 5AS-3, CS del 5BS-6, and CS Del 5DS-2 (Endo and Gill, 1996), supplied by Dr. B. Gill (Kansas State Univ.). These lines have distal deletions on their respective short arms, involving the hardness-related loci under study (Gill et al., 1996); (ii) Chromosome group V substitution lines of cultivars Cheyenne, Hope, Red Egyptian, Thatcher, and Timstein. Dr. B. Gill (Kansas State Univ.) provided the seeds of these lines; (iii) 5A/5A$^m$ recombinant substitution line No. 25, carrying the *T. monococcum Pina-A$^m$1*, *Pinb-A$^m$1*, and *Gsp-A$^m$1* loci, provided by Dr. J. Dvorak and Dr. M-C. Luo (Univ. of California, Davis). This line has a recombination point between *Xabg705* and *XksuH8* and includes a 40-cM segment of the short arm of chromosome 5A$^m$ (Luo et al., 2000); and (iv) CS as control. According to the single kernel characterization system (SKCS), CS should be classified as a moderately hard genotype.

### Experimental Procedures

In order to detect natural deletions at the studied loci, restriction fragment length polymorphism (RFLP) markers were used to characterize the chromosome substitution lines listed above. Nuclear DNA was isolated from leaves, following the procedure described by Dvorak et al. (1988). Southern blots and hybridizations were performed as described by Dubcovsky et al. (1994). Sample DNAs were digested with *Alu* I, *Bam* HI, *Bgl* II, *Dde* I, *Dra* I, *Eco* RI, *Eco* RV, *Hae* III, *Hind* III, *Hinf* I, *Mbo* I, *Pvu* II, *Rsa* I, *Sty* I, *Taq* I, and *Xba* I restriction enzymes and screened for polymorphism at *Pina-D1*, *Pinb-D1*, and *Gsp-1* loci with clones pTa31 (Sourdille et al., 1996), pTam19B2 (Tranquilli et al., 1999), and pGsp (Rahman et al., 1994), respectively. Clone pTa31, which corresponds to the *Pina-D1* cDNA, was provided by Dr. P. Joudrier (INRA, Montpellier, France). Clone pGSP was supplied by Dr. S. Rahman (Division of Plant Industry, CSIRO, Australia). Absence of the corresponding grain texture related loci on each of the deletion lines was checked by RFLP analysis.

### Field Evaluations

On the basis of the differences observed by the RFLP analyses at the grain texture related loci, 10 genotypes were selected for field evaluation. They included the Timstein and Red Egyptian chromosome substitution lines, deletion lines, and the 5A/5A$^m$ recombinant substitution line. Chinese Spring was included as the control and the 11 genotypes were evaluated in two field trials performed at different environments, Tulelake and Davis, CA, USA. Tulelake is located in the North Intermountain region of California and has a Tulebasin Mucky Silty Clay Loam soil (fine, mixed, superactive, mesic Aquandic Endoaquolls), whereas Davis is in the Sacramento Valley and has a Yolo Loam soil (fine-silty, mixed, superactive, nonacid, thermic Mollic Xerofluvents).

On 20 Apr. 1999, lines were seeded in a randomized complete block design (RCBD) with thirteen blocks in the field at Tulelake. Experimental units consisted of single row plots, 3.6 m in length with rows spaced 0.3 m apart. Twenty-four seeds per plot were hand planted, which represents ≈10% of the normal seeding rate for commercial plots. Preplant fertilizer was applied 1 wk before planting at the rate of 107 and 135 kg ha$^{-1}$ of N and P, respectively, as ammonium phosphate (16N-20P-0K). On 11 June 1999, herbicide 2,4D-Dichlorophenoxyacetic acid, dimethylamine salt (46.8%) at the rate of 2.2 L ha$^{-1}$ was used to control broadleaf weeds. On 16 June 1999, herbicide Difenzoquat methyl sulfate (31.2%) was applied at the rate of 2.7 L ha$^{-1}$ to control wild oats. The experiment was irrigated as needed. Individual plots were hand-harvested on 9 September and threshed using a Vogel plot thresher.

On 11 Nov. 1999, lines were sown in Davis, in a RCBD with thirteen blocks. Experimental units consisted of two-row plots, 2.4 m in length with rows spaced 0.3 m apart. Twenty-four seeds per plot were tractor-planted. Following a crop of beans, preplant fertilizer was applied 1 wk before planting at the rate of 45 kg ha$^{-1}$ of N as ammonium nitrate (33N-0P-0K). Additional fertilizer was applied 65 d after planting at the rate of 63 kg ha$^{-1}$ of N as ammonium nitrate (34N-0P-K). Herbicides were not used and weeds were controlled by hand-hoeing. Individual plots were hand-harvested on 27 May 2000, and threshed using a Vogel plot thresher.

There were no fungicides applied at either location since no leaf rust infections were recorded for plots in either experiment, including CS. The dry climate at both locations and the low seeding rate did not contribute to favorable conditions for fungal diseases.

The two different experiments were harvested in different years and different locations, but for simplicity they will be referred as *environments* throughout the paper.

Kernel hardness index, kernel weight, and kernel moisture values were determined by a SKCS (Perten Model 4100) from the average of 300 grains. The SKCS calculates the hardness index using an algorithm developed by the USDA.

### Data Analyses

Hardness data from each environment were analyzed in a RCBD with 13 blocks. Analysis of variance for hardness was performed both within environments and combined across environments. Levene's test was used to confirm homogeneity of variances between environments. In the combined model, environment, genotype × environment (G × E), and blocks within environment were considered random while genotype was considered fixed. In the combined analysis, overall differences and mean comparisons among genotypes were tested using the G × E mean square as error term. Differences between environments were tested using linear combinations of mean squares [(MS environment + MS error)/(MS environment × genotype + MS block within environment)] and effective degrees of freedom (Satterthwaite, 1946).

To study the potential effect of differences in kernel weight and kernel moisture on grain texture, the previous statistical analyses were repeated with the inclusion of these two measurements as covariates (ANCOVA). Homogeneity of regression coefficients among genotypes was tested before the ANCOVA analyses. Sixteen of the 286 data points were missing because of insufficient seed for the SKCS analyses. Therefore, least square means were used for the comparisons among genotypes. Least square means for genotypes were compared

with the CS control using Dunnett-Hsu test. All statistical analyses were performed using SAS 8.0 (SAS Institute, 2001).

## RESULTS

### Genetic Characterization

As expected, hybridization of DNA from CS with RFLP probe GSP showed three fragments with most of the restriction enzymes used in the present study, and only one fragment with RFLP probes for each of the puroindoline loci, that is, *Pina-D1* and *Pinb-D1*.

Each of the GSP fragments observed in CS was absent in one of the deletion lines 5AS-3, 5BS-6, and 5DS-2, confirming that the *Gsp-1* loci are distal to the deletion points in these three deletion lines. Hybridization of the same DNAs with RFLP probes for the *Pina-D1* and *Pinb-D1* loci revealed only one fragment that was absent in deletion line 5DS-2. Therefore, it is possible to use deletion lines 5AS-3 and 5BS-6 to study the effect of the deletion of the *Gsp-A1* and *Gsp-B1* loci on grain texture. Deletion line 5DS-2 was used to test the effect of the simultaneous deletion of *Gsp-D1*, *Pina-D1*, and *Pinb-D1* loci.

RFLP analyses with 16 restriction enzymes detected some polymorphisms between CS and the chromosome substitution lines analyzed in this study. Locus *Gsp-A1* was polymorphic in substitution lines CS (Timstein 5A), CS (Cheyenne 5A), and CS (Red Egyptian 5A) relative to CS with restriction enzymes *Bam* HI, *Bgl* II, *Eco* RV, *Hin*d III, and *Xba* I. Chinese Spring had a 4.5 kb *Xba* I fragment for *Gsp-A1* while all these three substitution lines had a larger 5.5 kb fragment (Fig. 1).

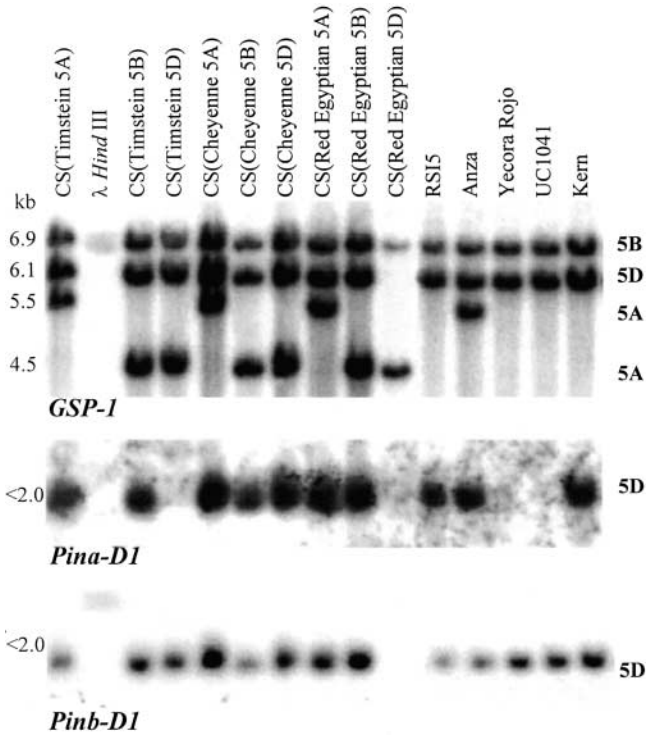


**Fig. 1. DNAs from Chinese Spring chromosome substitution lines and Californian wheat varieties digested with restriction enzyme *Xba* I. The same Southern blot membrane was hybridized with probes for *Gsp-1*, *Pina-D1*, and *Pinb-D1* genes.**

No differences were detected among *Gsp-B1* alleles with the selected restriction enzymes. The only difference detected for *Gsp-D1* was the presence of a deletion in CS (Red Egyptian 5D). This substitution line had deletions at the *Pina-D1* and *Pinb-D1* loci (Fig. 1). Substitution line CS (Timstein 5D) showed a single deletion for *Pina-D1*. This difference between CS (Red Egyptian 5D) and CS (Timstein 5D) was used to study the relative effect of the simultaneous deletion of the three loci relative to the deletion of *Pina-D1* on grain texture.

The presence of multiple deletions in the three hardness-related genes in Red Egyptian differs from the result presented by Morris et al. (2001). These authors made a survey of the *Pina-D1* and *Pinb-D1* loci in a large set of cultivars and indicated that Red Egyptian had only a deletion at the *Pina-D1* locus. This suggests that the accession used to produce the substitution lines of Red Egyptian differed from the one included in Morris et al. (2001).

The translocation of the *T. monococcum* segment of chromosome $5A^mS$ in the CS recombinant substitution line was detected with probes for the *Gsp-*$A^m$*1*, *Pina-*$A^m$*1*, and *Pinb-*$A^m$*1* genes using restriction enzyme *Hind III*. These three loci were reported to be present and tightly linked on the distal end of chromosome $5A^m$ in another accession of *T. monococcum* (Tranquilli et al., 1999).

Four hexaploid varieties, currently grown in a large area of California Central Valley, and one advanced breeding line were also included in the screening membranes and are reported here because of the presence of a deletion in the *Gsp-1* locus. These varieties were not included in the field trial that was limited to substitution and deletion lines in the isogenic background of CS. Cultivars 'RSI5', 'Yecora Rojo', 'Kern', and breeding line UC1041 showed a missing *Gsp-1* restriction fragment with most of the restriction enzymes, which was assigned to the *Gsp-A1* locus. This allelic variant is not present among the substitution lines (Fig. 1) and has not been reported previously in germplasm collections. No polymorphism was observed in *Gsp-B1* and *Gsp-D1* loci for this set of varieties. The deletion observed for *Pina-D1* was also present in Yecora Rojo and UC1041 (Fig. 1). Lines selected for field evaluation and their genotypes are summarized below.

### Grain Texture Variation among Genotypes

Hardness index values were significantly correlated ($P < 0.01$) with grain weight and grain moisture, but correlation values were small and explained only a small proportion of the variation in grain texture (grain weight $r = 0.40$; grain moisture $r = 0.31$). In order to eliminate a possible effect of the variation of these correlated traits on grain texture, the analysis of variance results were compared with analyses of covariance that included grain weight and grain moisture as covariates. The respective $P$ values are included in the last two columns of Tables 1 and 2.

Both types of analyses showed highly significant differences ($P < 0.0001$) in grain texture among genotypes

**Table 1. Statistical analysis of hardness index values for the two environments.**

| Source | df | Mean square | *F* value | ANOVA *P* | ANCOVA† Covariate: weight *P* | ANCOVA Covariate: moisture *P* |
|---|---|---|---|---|---|---|
| Genotype‡ | 10 | 6137.2 | 173.1 | <0.01 | <0.01 | <0.01 |
| 5D (del) vs. 5D (RE) | 1 | 41.3 | 1.2 | NS | NS | NS |
| 5D(T) vs. [5D (del) & 5D (RE)] | 1 | 290.0 | 8.2 | 0.02 | 0.02 | 0.02 |
| Environment§ | 1 | 2497.6 | 41.3 | <0.01 | NS | 0.02 |
| Environment × Genotype | 10 | 35.5 | 4.1 | <0.01 | <0.01 | <0.01 |
| Block (Environment) | 24 | 25.2 | 2.9 | <0.01 | <0.01 | <0.01 |
| Error | 213 | 8.6 | | | | |
| $R^2$ | | | | 0.974 | 0.981 | 0.974 |

† ANCOVA = analysis of covariance.
‡ Genotype effects were tested using the environment × genotype MS as the denominator in the *F* test.
§ Environment effects: *F* test = (MS environment + MS error)/[MS environment × genotype + MS block (environment)].

(Table 1). Differences in grain texture were also detected between the two environments but those differences were not significant when the grain weight was included as covariate (Table 1). This result indicates that differences in texture between both environments were partially related to differences in grain filling. Highly significant differences were detected in grain weight between environments ($P < 0.01$).

The interaction between genotypes and environments was also significant (Table 1), but its contribution to the total variation was small compared with the genotype effect. The complete model explained 97.4% of the total variation in grain texture present in this experiment. Partition of the variance components showed that the largest proportion of the total variation was explained by the variation among genotypes (87.4%) and between environments (8.5%). Only a small percentage of the variation was explained by the variation among blocks (0.6%) and by the G × E interactions (0.8%).

The addition of the grain weight as covariate increased the proportion of the variation explained by the model to 98.1% and reduced the variation among environments to nonsignificant levels. Adjustment of the means by grain moisture did not increase the proportion of the variation explained by the model (97.4%). This was expected based on the low correlation between texture and grain moisture.

The relative order of the genotypes based on hardness index values was almost identical for both environments (Fig. 2) as expected based on the low percentage of the variation explained by the G × E interaction (<1%). Therefore, mean comparisons were performed simultaneously for both environments (Table 2).

The genotypes with one, CS (Timstein 5D), or two deletions in the puroindoline genes [CS (Red Egyptian 5D) and deletion line 5DS-2; Table 2] were significantly different from CS in both the combined and in the separate environment analyses. The hardness indexes from these three varieties were ≈30 units higher than CS hardness index (Table 2). Deletion line 5DS-2 did not differ significantly from substitution line CS (Red Egyptian 5D). The adjusted means for the hardness index values of the two lines carrying two deletions in the puroindoline genes were significantly higher in all the analyses ($P = 0.02$) than the values observed in substitution line CS (Timstein 5D) carrying a single mutation in the *Pina-D1* gene (Table 1). A similar result was observed in the analysis for each of the individual environments.

The substitution line of the recombinant chromosome carrying the segment of chromosome $5A^m$ from *T. monococcum* was in the other extreme of the hardness spectrum. This line was significantly softer than CS in all the statistical analyses ($P < 0.01$) including both combined and individual environment analyses. This line showed hardness index values ≈9 units smaller than the CS control (Table 2).

Substitution line CS (Timstein 5B) showed hardness index values slightly smaller than CS (average 6 units, $P = 0.04$). These differences were not significant in the combined analysis after correction with the grain weight in the covariance analysis ($P = 0.21$, Table 2). Differences between these two lines were significant ($P < 0.01$) in the individual environments in both the ANOVA and ANCOVA analyses due to the smaller error term of these analyses. Therefore, additional studies will

**Table 2. Comparison between the adjusted means (± standard error) of the individual genotypes with Chinese Spring using the DUNNETT test (environment × genotype MS as error term). Genotypes are arranged by decreasing adjusted hardness index means. Presence of an RFLP fragment for each gene in the A, B, and D genomes is indicated by a '+' and absence by a '−'.**

| *Gsp* ABD | *Pina* ABD | *Pinb* ABD | Genotype | ANOVA | *P* | ANCOVA Weight | *P* | ANCOVA Moisture | *P* |
|---|---|---|---|---|---|---|---|---|---|
| +++ | −−+ | −−+ | 'Chinese Spring' | 61.3 ± 1.2 | | 61.7 ± 1.2 | | 61.2 ± 1.3 | |
| ++− | −−− | −−− | CS (Red Egyptian 5D) | 95.6 ± 1.2 | <0.01 | 93.8 ± 1.3 | <0.01 | 95.9 ± 1.6 | <0.01 |
| ++− | −−− | −−− | Deletion 5DS-2 | 93.7 ± 1.3 | <0.01 | 92.8 ± 1.3 | <0.01 | 93.9 ± 1.4 | <0.01 |
| +++ | −−− | −−+ | CS (Timstein 5D) | 90.4 ± 1.2 | <0.01 | 89.2 ± 1.2 | <0.01 | 90.7 ± 1.5 | <0.01 |
| +−+ | −−+ | −−+ | Deletion 5BS-6 | 62.6 ± 1.3 | 0.99 | 61.8 ± 1.3 | 1.00 | 62.4 ± 1.4 | 0.99 |
| +++ | −−+ | −−+ | CS (Red Egyptian 5B) | 61.0 ± 1.2 | 1.00 | 62.2 ± 1.3 | 1.00 | 60.7 ± 1.4 | 1.00 |
| −++ | −−+ | −−+ | Deletion 5AS-3 | 59.9 ± 1.2 | 0.97 | 57.9 ± 1.3 | 0.34 | 59.7 ± 1.3 | 0.97 |
| +++ | −−+ | −−+ | CS (Red Egyptian 5A) | 59.7 ± 1.2 | 0.93 | 59.7 ± 1.2 | 0.85 | 59.6 ± 1.3 | 0.95 |
| +++ | −−+ | −−+ | CS (Timstein 5A) | 59.2 ± 1.2 | 0.79 | 59.5 ± 1.2 | 0.77 | 59.1 ± 1.2 | 0.83 |
| +++ | −−+ | −−+ | CS (Timstein 5B) | 55.4 ± 1.2 | 0.04 | 57.5 ± 1.3 | 0.21 | 55.3 ± 1.2 | 0.05 |
| +++ | +−+ | +−+ | $5A/5A^m$ recombinant | 52.7 ± 1.5 | <0.01 | 52.7 ± 1.5 | <0.01 | 52.6 ± 1.5 | <0.01 |

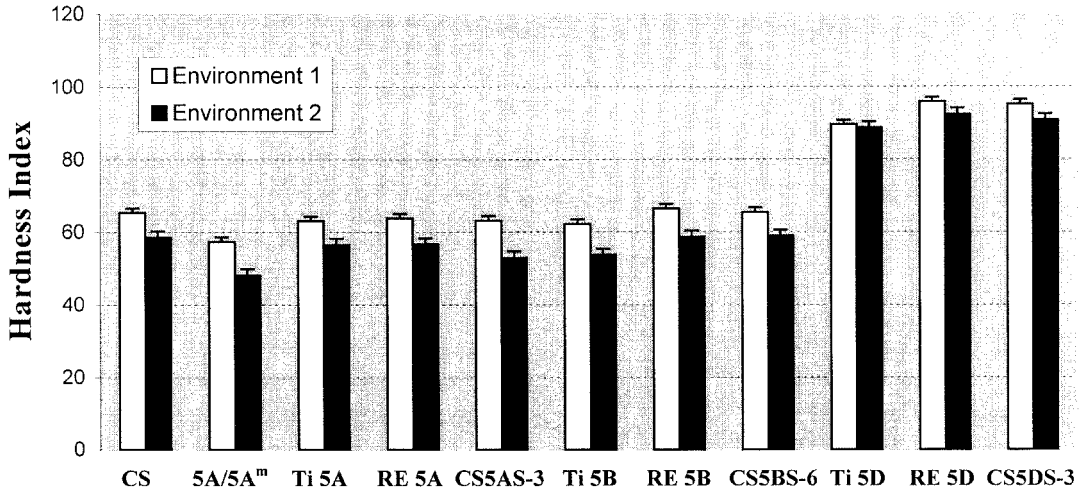


**Fig. 2. Average single kernel characterization system (SKCS) hardness index values adjusted by grain weight. Chromosome substitution lines are indicated by the Chinese Spring (CS) substituted chromosome and the donor cultivar (Ti = Timstein, RE = Red Egyptian). 5A/5A$^m$ indicates the CS/*Triticum monococcum* recombinant chromosome substitution line. Error bars indicate 95% confidence intervals.**

be necessary to confirm this effect, and also to determine if this difference is caused by a different *Gsp-B1* allele or by differences in other regions of the Timstein 5B chromosome.

## Genotype by Environment Interactions Within Hard and Moderately Hard Classes

The G × E interactions explained only 1% of the variation in hardness index values in the analysis including all genotypes (Table 1). A different result was observed when two separate analyses were performed for the hard-texture genotypes [CS (Timstein 5D), CS (Red Egyptian 5D), and CS Deletion 5DS-2] and for the other seven genotypes.

Partition of the variance components in the ANOVA for the three hard-texture genotypes revealed that the genotypic differences between the lines with single and double puroindoline deletions accounted for 23.1% of the total variation whereas the differences between environments accounted for 28.8% of the variation. Also, a large component of the environmental variance (58.9%) was observed in the ANOVA for the seven moderately hard-texture genotypes. Genotypic differences among these lines accounted for only 18.3% of the total variation. The genetic variation component was significantly smaller in both separate analyses by texture class than in the analysis including both classes.

## DISCUSSION

Nonsignificant differences on grain texture were detected between the hardness indexes of the CS control and CS Del 5AS-3 or CS Del 5BS-6 deletion lines, in spite of the large number of blocks included in the study. In addition, the RFLP polymorphisms observed in Red Egyptian and Timstein *Gsp-A1* locus were not correlated with significant differences in grain texture. These results suggest that these two *Gsp-1* loci do not have a determinant role on grain texture. It would be interesting to test if the slight effect showed by the CS (Timstein 5B) substitution line is linked to variation in the *Gsp-B1* locus or to other genes in Timstein chromosome 5B.

The differential effect of the *Gsp-1* and puroindoline loci on grain texture parallels their similarities in protein sequence. Puroindoline a and b proteins are more similar to each other (67.5% similar) than to the GSP protein [57 and 58% similar, respectively; Gautier et al., 1994; Rahman et al., 1994; and our comparisons using ClustalW (unpublished data, 2002)].

Absence of the *Pina-D1* transcript or mutations of the *Pinb-D1* product are associated with hard-textured phenotypes (Giroux and Morris, 1998; Morris et al., 2001). We show in this study that the simultaneous deletion of both puroindoline loci results in average hardness indexes ≈5% higher than those observed in genotypes with individual mutations. This result suggests that mutation of one puroindoline locus is not enough to completely eliminate the effect of the other on grain texture.

It is interesting to point out that the hexaploid Red Egyptian 5D substitution in CS has an identical puroindoline composition as tetraploid wheats (no puroindoline genes present). Therefore, the Red Egyptian double deletion offers the possibility to engineer durum-like hardness in hexaploid wheat. The combination of the double puroindoline deletion with genes for yellow semolina color, white grain color, and low polyphenol oxidase activity may open the way for the development of hexaploid varieties with acceptable pasta quality.

The incorporation of additional copies of puroindoline genes in the substitution line with the recombined 5A/5A$^m$ chromosome produced the opposite effect to the elimination of puroindoline genes in the substitution lines described above. The presence of the *Pina-A$^m$1* and *Pinb-A$^m$1* loci from *T. monococcum* was associated with a decrease of 14% in the mean hardness index value compared with the control CS, and with the lowest hardness index value among all the analyzed genotypes (Table 2). This result indicates that the *T. monococcum* genes can be expressed in the hexaploid background

and that they can act additively to the puroindoline genes present in chromosome 5D.

In this study, the effect of the *T. monococcum* genes was determined in the moderately hard germplasm CS. The same *T. monococcum* genes are currently being introgressed into soft wheat varieties to test their value to modulate soft grain textures.

The usefulness of the *T. monococcum* puroindoline genes in breeding programs might be limited if the introduced chromosome segment from this species has any detrimental effect on agronomic performance. Although this aspect should be further evaluated, in the present study no differences in grain weight were observed between the 5A/5A$^m$ recombinant line (29.2 g) and the CS control (29.5 g). We have initiated a project aimed to further reduce the 5A$^m$S segment by additional cycles of recombination in the presence of the *ph1b* mutation.

The puroindoline genes present in the *T. monococcum* segment have been transferred to the 5A chromosome and therefore they would be easier to transfer to a tetraploid background than the puroindoline genes present on chromosome 5D. However, it is difficult to predict at this point the effect of the *T. monococcum* puroindoline genes on grain texture in pasta wheats, and therefore difficult to predict the possible applications of such genotypes.

The Red Egyptian and *T. monococcum Ha* loci containing lower and higher number of active puroindoline genes may become useful tools to modulate grain hardness within both the hard-textured and soft-textured hexaploid wheat varieties.

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