Wheat Grain Hardness Among Chromosome 5D Homozygous Recombinant Substitution Lines Using Different Methods of Measurement

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ABSTRACT

The level of grain hardness of wheat (*Triticum aestivum*) cultivars profoundly affects milling properties and end-use. We examined grain hardness among a genetically defined set of 83 chromosome 5D homozygous recombinant substitution lines derived from soft wheat cv. Chinese Spring and hard wheat cv. Cheyenne and compared four common methods of measuring wheat grain hardness. Measures of grain hardness included a modified particle size index, Brabender Quadrumat flour milling, near-infrared reflectance (NIR) spectroscopy, and the single-kernel characterization system (SKCS). Duncan's multiple range test was used to group recombinant lines according to parental classes. Quadrumat milling fractions, percent bran and middlings, were well correlated to NIR and SKCS grain hardness, whereas break flour, a traditional measure of grain hardness, was poorly correlated to other hardness measures. NIR and SKCS grain hardness measures provided the greatest and similar mean separations. Both methods identified recombinant lines as being significantly outside either parental class and significantly different from and in between the two parental classes. Between two divergent environments, correlations (*r*) for Quadrumat bran and middlings percents and NIR and SKCS hardness ranged from 0.83 to 0.94. Analysis of variance indicated that lines differed substantially for hardness, and hardness was highly influenced by environment, albeit consistently, as indicated by low line-location model interaction terms. The results confirmed the presence of major allelic differences assignable to chromosome 5D and suggested the action of minor gene(s). Break flour, in particular, showed strong indications of transgressive segregation independent of the *Hardness* (*Ha*) locus. The Perten 4100 SKCS provided the best (most discriminating) measure of the material properties of the wheat endosperm manifested by the action of the *Ha* locus.

Grain hardness has a profound influence on milling performance and end-use wheat (*Triticum aestivum* L.) quality. Grain hardness affects energy consumption and design approaches to flour milling (e.g., number of roll stands and sifting area) and particle size distribution and starch damage level of flours and, consequently, end-product performance and quality (Pomeranz and Williams 1990, Morris and Rose 1996).

Cultivars of common hexaploid ($2n=42=6X=$AABBDD) wheat generally are separated into two distinct classes of hardness: soft and hard. The distinction serves as the primary basis of wheat cultivar classification (e.g., hard red winter vs. soft red winter) in the United States and elsewhere (FGIS 1996) and illustrates its importance in the value, marketing, and use of wheat.

The classification of wheat cultivars into two hardness categories is consistent with a single major gene, *Hardness* (*Ha*), on the short arm of chromosome 5D (Mattern et al 1973, Law et al 1978). The expression of this gene is poorly understood, although recent work indicates it is a complex locus that codes for two related 15-kDa proteins: puroindoline a and puroindoline b (Morris et al 1994; Giroux and Morris 1997, 1998). Puroindoline a and b comprise what has been referred to previously as friabilin, which are proteins complexed with bound polar lipids that are abundant on the surface of starch washed from soft, but not hard, wheat (Greenwell and Schofield 1986; Greenblatt et al 1995; Giroux and Morris 1997, 1998).

Since its discovery, among the hundreds of genotypes evaluated, no exception to the "soft wheat-abundant friabilin" rule has been reported. However, the phenomenon of friabilin occurrence is more complicated than it might initially appear, and no cause-and-effect relationship between friabilin and grain hardness has been established. One fact that must be accommodated in any model of grain hardness is that friabilin is present in similar amounts in both soft and hard endosperm (Jolly et al 1993, Greenblatt et al 1995). Therefore, differences in friabilin levels per se are not the cause of grain hardness. Only when starch is isolated aqueously does the soft-hard difference in starch surface friabilin manifest itself. In a related article (Giroux and Morris 1997), we reported a change in the nucleotide and amino acid sequence of puroindoline b from hard wheat cvs. Cheyenne and Wanser that may explain why friabilin in hard wheat has reduced affinity for the starch granule surface, and possibly polar lipids. Among the same set of chromosome 5D homozygous recombinant substitution lines used here, Giroux and Morris (1997) found no recombination between the puroindoline b sequence change and grain hardness.

The endosperm of wheat is composed primarily of starch granules surrounded by a continuous protein matrix (Bradbury et al 1956, Barlow et al 1973). Starch is synthesized in the amyloplast, an organelle bounded by a typical membrane lipid bilayer. Multiple starch granules are present in each cell. When physiologically mature, wheat grain undergoes preprogrammed cell death and desiccation. The manner in which desiccation occurs appears to be related to the manifestation of grain hardness. Bechtel et al (1996) found that developing kernels of both soft and hard wheat were soft if freeze-dried. When air-dried, the difference in hardness between soft and hard wheat was manifested.

When kernels of soft wheat are crushed, fracture planes tend to occur at the starch granule-matrix protein interface, and many free starch granules are released. Soft wheat flours have smaller mean particle size compared with hard wheat flours. In contrast, when kernels of hard wheat are crushed, fracture planes more frequently pass through the starch granule, resulting in broken granules, fewer free starch granules, and larger particles (Hoseney and Seib 1973). The results stem from fundamental differences in the material properties of soft and hard wheats. Because the basic components of soft and hard wheat endosperm are essentially identical (Barlow et al 1973), differences in adhesion among heterogeneous endosperm components likely explain differences in grain hardness.

Regardless of the specific underlying mechanism(s) involved, nearly all means of measuring wheat grain hardness are empirical and rely on manifestations of the material properties of the endosperm or whole kernel. Measurement methods include 1) physical

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measures related to crushing, deformation, and fracturing of the kernel; 2) time or energy required to reduce a grain sample to a specific particle size (usually determined by the aperture of a screen); 3) particle size distribution of meal or flour obtained after grinding; 4) quantity or degree of mechanically damaged starch after grinding; and 5) what can be described broadly as milling properties: usually break flour release or reduction characteristics of middlings stocks (MacRitchie 1980, Pomeranz and Williams 1990, Anjum and Walker 1991, Glenn et al 1991).

All of the methods require that a kernel be loaded to the point of mechanical failure, often at fairly rapid rates ($\ll$1 sec). Not all of the methods of assessing grain hardness exert the same type of mechanical stress, although compression and shear commonly occur in all. Glenn et al (1991) provided an elegant engineering approach to studying the material properties of wheat endosperm in the context of hardness. However, the procedure, which used submillimeter-sized endosperm cylinders prepared from individual kernels, is arduous and not amenable to large sample numbers.

In this report, we examine the expression of wheat grain hardness and compare four common methods of determining hardness using a genetically defined set of chromosome 5D homozygous recombinant substitution lines derived from soft cv. Chinese Spring and hard cv. Cheyenne grown under two diverse environments.

MATERIALS AND METHODS

Genetic Materials

Homozygous chromosome 5D recombinant substitution lines were developed from the soft red spring wheat cv. Chinese Spring and hard red winter wheat cv. Cheyenne by R. S. Kota and J. Dvorak (University of California-Davis) following the procedure of Law (1966). Each line contains 20 pairs of normal euploid Chinese Spring and one pair of recombined Chinese Spring/Cheyenne 5D chromosomes in a homozygous state. The procedure involved crossing Chinese Spring with the Chinese Spring/Cheyenne 5D substitution line, crossing the resultant F_1 as male to Chinese Spring monosomic for 5D, recovering monosomic progeny carrying a recombinant chromosome, and selfing the progeny to fix the recombined chromosome in a homozygous state.

Grain samples were produced in field plots at the University of California Research Center, Tule Lake, in 1991 and at the Spillman Agricultural Farm, Washington State University, Pullman, in 1994. Two randomized replicate plots were grown per location in a completely randomized design. Chinese Spring (CS) and the Chinese Spring/Cheyenne 5D whole chromosome substitution (CS(CNN5D)) line were grown as parental controls. Days to heading, calculated from the planting date, were recorded for samples grown at Pullman.

TABLE I
General Grain Quality of Parents and 83 Homozygous Chromosome 5D Recombinant Substitution Wheat Lines Derived from Chinese Spring (CS) and Chinese Spring Substitution Cheyenne 5D [CS(CNN5D)] Grown at Two Locations

Location	Test Weight (lbs/bu)	Protein Content (%)	Kernel Weight (mg)	Kernel Size (mm)
Tule Lake, CA				
CS	60.7	13.8	31.6	2.67
CS(CNN5D)	59.9	14.5	30.7	2.69
Recombinant lines				
Mean	59.9	14.4	31.3	2.74
Range	56.4–62.2	12.6–16.6	27.3–35.0	2.52–2.97
Pullman, WA				
CS	58.0	15.8	23.1	2.25
CS(CNN5D)	57.1	17.2	22.2	2.25
Recombinant lines				
Mean	56.4	16.7	22.8	2.29
Range	52.7–59.6	14.8–18.6	19.6–27.1	2.07–2.49

Sample Preparation and Analysis

Grain samples were cleaned with an air aspirator (Kice Metal Products Co., Wichita, KS). Test weight was determined using cleaned grain and expressed as pounds per Winchester bushel using a 90-cm^3 cup. The grain was then scoured (Forster Manufacturing Co., Ada, OK). A 20-g subsample was ground to pass through a 0.5-mm screen with a Udy (Ft. Collins, CO) grinder and used to estimate protein content with a near-infrared reflectance (NIR) spectrometer (model 450, Technicon, Tarrytown, NY).

Four individual aliquots from Tule Lake and one aliquot from Pullman were loaded and measured. Nitrogen content was determined on ≈0.25-g aliquots of ground sample using the Dumas combustion method (model FP-428, Leco Corp., St. Joseph, MI; Approved Method 46-30 [AACC 1995]) and converted to percent protein by multiplying by 5.7. These nitrogen measurements were used to verify the protein values obtained with NIR. Starch damage was measured in duplicate using an enzyme digestion assay procedure (Megazyme International, Wicklow, Ireland) as described by Gibson et al (1992) (Approved Method 76-31 [AACC 1995]) (Tule Lake only).

Measurement of Grain Hardness

Grain hardness was determined by four procedures: particle size fractions of Udy-ground meal, Brabender Quadrumat milling, NIR spectroscopy of Udy-ground meal, and a single-kernel characterization system. Particle size fractions were determined using 3 g of Udy-ground meal sifted sonically (ATM Corp., Milwaukee, WI). The weight of material remaining on the no. 70 (200 µm) sieve, the no. 200 (74 µm) sieve, and the fines collector (<74 µm) was determined and expressed as a percentage of the total.

TABLE II
Analysis of Variance (ANOVA) of Four Methods of Determining Wheat Grain Hardness Among Homozygous Chromosome 5D Recombinant Substitution Wheat Lines Derived from Chinese Spring and Chinese Spring Substitution Cheyenne 5D[a]

Method	ANOVA Whole Model (*F*)			Tule Lake, CA		Pullman, WA	
	Lines	Locations	Line-Location	*F*	R^2	*F*	R^2
Sonic sifter whole-grain meal fraction							
Coarse (>200 µm)	…	…	…	1.00 ns	0.50	…	…
Medium (74–200 µm)	…	…	…	1.97**	0.66	…	…
Fine (<74 µm)	…	…	…	1.41 ns	0.58	…	…
Quadrumat milling fraction							
Bran (>500 µm)	29.22***	63.23***	1.62**	12.53***	0.93	19.46***	0.95
Middlings (150–500 µm)	11.19***	26.17***	1.13 ns	3.59***	0.78	11.76***	0.92
Break flour (<150 µm)	6.37***	1.55 ns	1.00 ns	2.25***	0.69	6.67***	0.87
Near-infrared reflectance spectroscopy	40.71***	16.79***	1.73**	14.05***	0.93	39.33***	0.97
Single-kernel characterization system	46.17***	30.36***	1.66***	33.24***	0.97	19.64***	0.95

[a] Whole model composed of lines, locations, and line-location interactions and a lines-only model for each of two locations. ns, *, **, and ***, indicate P > 0.05, = 0.01–0.05, = 0.001–0.01, and < 0.001, respectively.

A 50-g sample of grain was equilibrated to 13.5% moisture content in an elevated relative humidity chamber and milled on a Brabender Quadrumat Jr. experimental mill following the general procedure of Jeffers and Rubenthaler (1977). Mill output was separated into bran, middlings stock (midds), and break flour fractions using 35-mesh (500 µm) and 100-mesh (150 µm) test sieves (W.S. Tyler, Mentor, OH). Fraction yield was calculated as a percentage of total products.

The NIR spectrometer described above was used to assess grain hardness of Udy-ground sample aliquots used for protein measurement (four individual aliquots per replicate for Tule Lake, one aliquot for Pullman). For Tule Lake samples, two operators each took two separate readings. NIR hardness is a dimensionless value based on a calibration derived from a set of wheat grain samples of known hardness (Approved Method 39-70A [AACC 1995]).

A 300-kernel sample was analyzed for hardness with a commercial prototype of the single-kernel characterization system (SKCS) (model 4100, Perten Instruments, Springfield, IL) as described by Martin et al (1993). In addition to individual grain hardness, the instrument also measured individual kernel weight and size (outer dimension). All grain and flour parameters are reported on an "as-is" moisture basis, ≈9.5–10.5% for grain and ≈12.5–13.5% for flour.

Statistical Analyses

The four separate repeated measures of grain protein using NIR were averaged for statistical analyses (Tule Lake only). The general linear models procedure of the Statistical Analysis System (SAS Institute, Cary, NC) was used to conduct analysis of variance (ANOVA) within and across locations, considering all factors as random. Simple correlation coefficients were similarly calculated across and within locations using correlation procedures (SAS). Completely randomized design, two-replicate field plots were used; mean separation of lines was determined by Duncan's multiple range test (DMRT) at $\alpha = 0.05$.

RESULTS

General Performance of Lines

The 83 chromosome 5D recombinant substitution lines derived from CS and CS(CNN5D) performed relatively well at Tule Lake but suffered at Pullman due to hot, dry weather during mid-to-late grain fill. The general grain quality characteristics test weight, protein content, and kernel weight and size are presented in Table I. Each of these four traits exhibited a relatively large range among recombinant lines within location: 6–7 lbs/bu test weight, 4% protein content, 7–8 mg kernel weight and ≈0.4 mm kernel size (Table I). For kernel weight and size, the two locations produced non-overlapping distributions. Even so, ANOVA showed that location was always a much greater source of variation compared with lines and location-line interaction (e.g., $F > 1{,}000$ for locations, versus <10 for other components) (data not shown).

Recombinant lines at Pullman exhibited distinct differences for heading date. A plot of the data indicated two parental classes: 78.0 days for CS and 85.5 days for CS(CNN5D) (data not shown). The earlier heading class contained 55 recombinants, and the later heading class contained 28 recombinants.

Methods of Measuring Wheat Grain Hardness

The first method of measuring hardness, sonic sifting of whole-grain meal, relies on differences in particle size distribution after grinding with a Udy mill. Separating meals into coarse, medium, and fine fractions was largely ineffective in differentiating hardness among recombinant lines from Tule Lake (Table II). The best discriminated fraction of the three appeared to be the medium fraction (74–200 µm). Mean and standard deviation values for the parental types were 32.1 ± 1.5 for CS and 35.2 ± 1.6 for CS(CNN5D). The mean and range for recombinants were 33.0, and 24.1–40.5, respectively. Due to its poor performance and technical difficulties in operating the instrument, sonic sifting was not conducted on samples grown at Pullman.

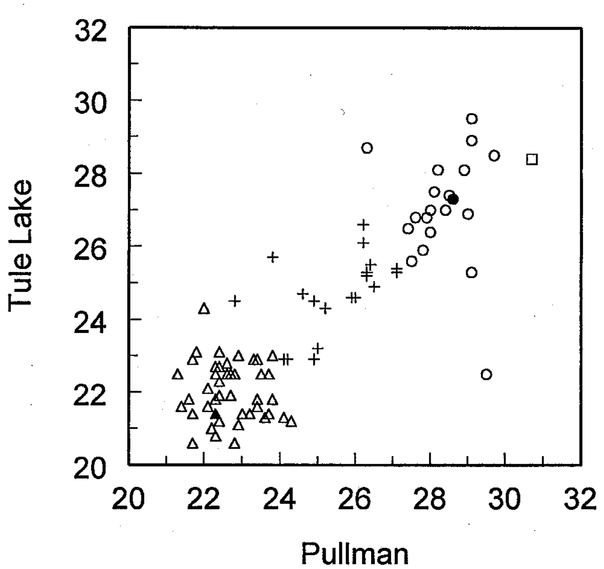

Fig. 1. Quadrumat milling fraction percent bran yield of 83 homozygous chromosome 5D recombinant substitution wheat lines derived from Chinese Spring (CS) and substitution line Chinese Spring (Cheyenne 5D) (CS(CNN5D)) grown at Pullman, WA, and Tule Lake, CA. Parents and recombinant lines are denoted as: CS: ●; CS(CNN5D): ▲; lines not significantly different from CS: ○; lines not significantly different from CS(CNN5D): △; lines significantly greater than CS: □; and lines significantly different from and in between the two parental classes: +. Duncan's multiple range test parental class limits were 26.5–29.3 for CS and 21.1–23.4 for CS(CNN5D).

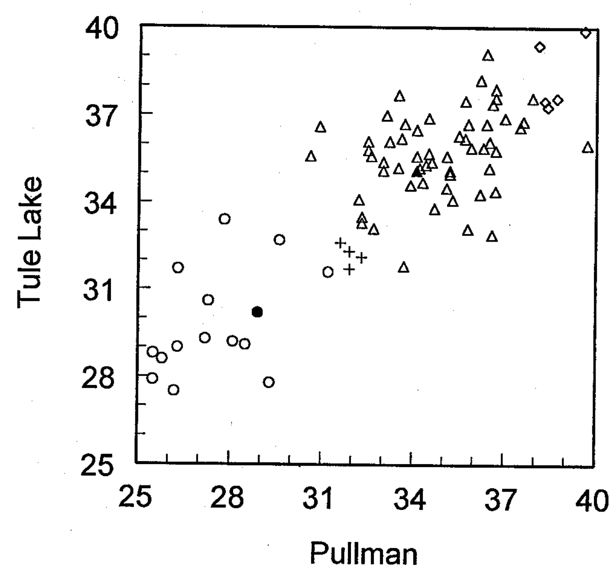

Fig. 2. Quadrumat milling fraction percent middlings yield of 83 homozygous chromosome 5D recombinant substitution wheat lines derived from Chinese Spring (CS) and substitution line Chinese Spring (Cheyenne 5D) (CS(CNN5D)) grown at Pullman, WA, and Tule Lake, CA. Parents and recombinant lines are denoted as: CS: ●; CS(CNN5D): ▲; lines not significantly different from CS: ○; lines not significantly different from CS(CNN5D): △; lines significantly greater than CS(CNN5D): ◇; and lines not significantly different from both parents: +. Duncan's multiple range test parental class limits were 26.7–32.2 for CS and 31.8–37.8 for CS(CNN5D).

The second method, Quadrumat milling, also relies on differences in particle size distribution but uses a small-scale flour milling operation to effect grinding. Of the fractions produced by milling and sieving with a Quadrumat mill, the bran fraction showed the greatest discrimination among lines and also was highly influenced by location (Table II). Percent bran exhibited a significant but small line-location interaction. Bran percent for CS was 27.3 ± 0.4 and 28.6 ± 3.0 for Tule Lake and Pullman, respectively, and for CS(CNN5D) was 21.4 ± 0.5 and 22.3 ± 0.1, respectively. In the combined location analysis, DMRT identified one line with greater bran than CS, 19 in the CS parental class, 19 different from and in between both parents, and 44 in the CS(CNN5D) parental class (Fig. 1). The correlation between the two locations was $r = 0.90$.

The midds Quadrumat milling fraction was the second best for differentiating hard-soft recombinant lines (Table II). CS had 30.2 ± 0.3% and 28.9 ± 2.1% midds at Tule Lake and Pullman, respectively. CS(CNN5D) had 35.1 ± 3.4% and 34.1 ± 1.4% midds at Tule Lake and Pullman, respectively. ANOVA of percent midds using DMRT on combined analysis indicated that five lines had a greater percentage of midds than CS(CNN5D). The remaining 78 lines were similar to one parent or the other, with four lines not significantly different than either parent (Fig. 2). There were 18 lines not significantly different than CS. The correlation for midds between locations was $r = 0.83$.

Of the three milling fractions, Quadrumat break flour percent produced the least differentiation among recombinant lines (Table II). The two parental values were relatively similar and not significantly different from one another (Fig. 3; data not shown). The overall mean for CS was 42.6% and for CS(CNN5D) was 43.6%. DMRT groupings for the two parental types nearly completely overlapped for each individual location and the combined data set (Fig. 3). However, in the combined ANOVA, 18 recombinant lines were identified as having significantly less break flour than the parent with the least break flour, CS (Fig. 3). Graphically, the recombinant lines appeared to fall into two groups: one containing the CS(CNN5D) high break flour group; the other containing the lines with less break flour than CS (Fig. 3). The seven lines that were significantly lower than CS(CNN5D) but not different from CS were approximately equally divided between the two groups. The correlation for percent break flour between locations was $r = 0.74$.

The third means of measuring wheat grain hardness, NIR, also is based on particle size distribution of ground whole-grain meal but relies on spectral characteristics of the sample, as opposed to physical separation by sieving. NIR spectroscopy produced highly significant F values for both lines and locations (Table II). The interaction term also was significant, albeit at a much lower level (Table II, whole model). ANOVA for lines alone at each location produced highly significant F values and high model R^2 values (Table II). The distribution of NIR hardness values for lines at each location are presented in Fig. 4 as a plot of the hardness value obtained at each location for each recombinant line. CS had mean NIR hardness values of 39.6 ± 0.6 and 36.2 ± 0.4 for Tule Lake and Pullman, respectively. CS(CNN5D) had mean hardness values of 82.0 ± 19.2 and 81.6 ± 4.8 for Tule Lake and Pullman, respectively.

The combined location ANOVA for the two locations placed 19 lines in the CS parental class and 49 lines in the CS(CNN5D) parental class. One line was identified as being harder than CS(CNN5D), and 14 lines were significantly different from or in between the two parental classes. The results are presented in Fig. 4 and are consistent across locations in that no line ever appeared in one parental class at one location and the other parental class at the other location. This consistency is reflected in the relatively low ANOVA interaction F value (Table II). The correlation between the two locations was $r = 0.92$.

The fourth method of assessing wheat grain hardness, SKCS, exhibited the greatest overall mean separation and highest F value for lines (whole model) (Table II). Similar to NIR hardness, location effects were highly significant and line-location interaction was significant, but at a much lower level. Individual locations exhibited high F values and model R^2 values (Table II). SKCS hardness values for the parents were 58.8 ± 0.3 and 59.9 ± 5.4 for CS at Tule Lake and Pullman, respectively, and 78.3 ± 1.2 and 79.4 ± 0.6 for CS(CNN5D) at Tule Lake and Pullman, respectively. DMRT produced the largest number of discrete classes for

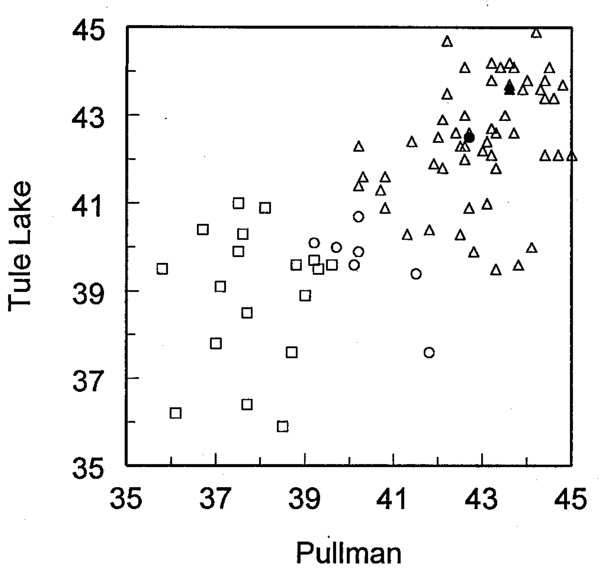

Fig. 3. Quadrumat milling fraction percent break flour yield of 83 homozygous chromosome 5D recombinant substitution wheat lines derived from Chinese Spring (CS) and substitution line Chinese Spring (Cheyenne 5D) (CS(CNN5D)) grown at Pullman, WA, and Tule Lake, CA. Parents and recombinant lines are denoted as: CS: ●; CS(CNN5D): ▲; lines not significantly different from both parents: △; lines not significantly different from CS but less than CS(CNN5D): ○; and lines significantly less than both parents: □. Duncan's multiple range test parental class limits were 39.6–44.9 for CS and 40.8–44.9 for CS(CNN5D).

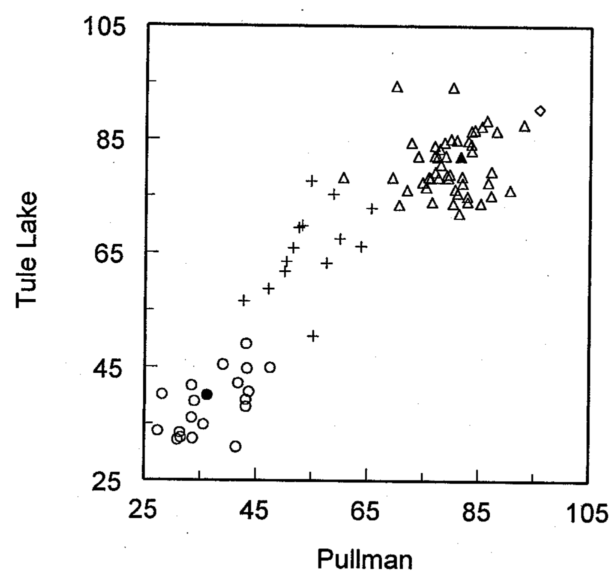

Fig. 4. Near-infrared reflectance hardness of 83 homozygous chromosome 5D recombinant substitution wheat lines derived from Chinese Spring (CS) and substitution line Chinese Spring (Cheyenne 5D) (CS(CNN5D)) grown at Pullman, WA, and Tule Lake, CA. Parents and recombinant lines are denoted as: CS: ●; CS(CNN5D): ▲; lines not significantly different from CS: ○; lines not significantly different from CS(CNN5D): △; lines significantly harder than CS(CNN5D): ◇; and lines significantly different from and in between the two parental classes: +. Duncan's multiple range test parental class limits were 30.6–46.3 for CS and 71.9–90.4 for CS(CNN5D).

SKCS hardness data compared with other hardness measurement methods (Fig. 5; data not shown). Combined location analysis placed 13 lines in the CS parental class and 55 in the CS(CNN5D) parental class; one line was softer than the soft parent, three lines were harder than the hard parent, and 11 lines were in between the two parental classes (Fig. 5). The correlation between the two locations was $r = 0.94$.

Relationships Between Grain Hardness and Other Grain Traits

Simple correlations and scatter plots were the primary means of examining whether grain hardness was related to other traits. Comparing the three Quadrumat milling fractions with test weight, grain protein, and kernel weight and size produced correlations (r) of from –0.20 to 0.48; no single grain trait explained more than 23% of the total variation either within or across locations (data not shown). For the Pullman data, correlations between percent break flour yield and heading date or kernel size were significant; the highest observed ($r = 0.46$ and –0.53, respectively). Similarly, comparisons among NIR and SKCS hardness and test weight, grain protein, and kernel weight and size produced correlations (r) from –0.39 to 0.42 (data not shown).

Relationships Among Grain Hardness Measures

The four methods of measuring grain hardness were generally highly intercorrelated, and both locations produced nearly identical correlation statistics. Indeed, the correlation coefficient (r) for an individual location never deviated by more than 0.09 from that for the combined data set. Consequently, only the combined results are presented in Table III. Overall, NIR and SKCS hardness were the most highly correlated of all the hardness measurement methods, whereas break flour yield had the poorest correlation. NIR and SKCS hardness also were well correlated with percent bran and midds from Quadrumat milling. Percent midds also was reasonably well correlated with bran and break flour. To summarize the interrelationships among hardness measures, harder grain exhibited a lower percent yield of bran, a higher yield of midds, and higher NIR and SKCS values.

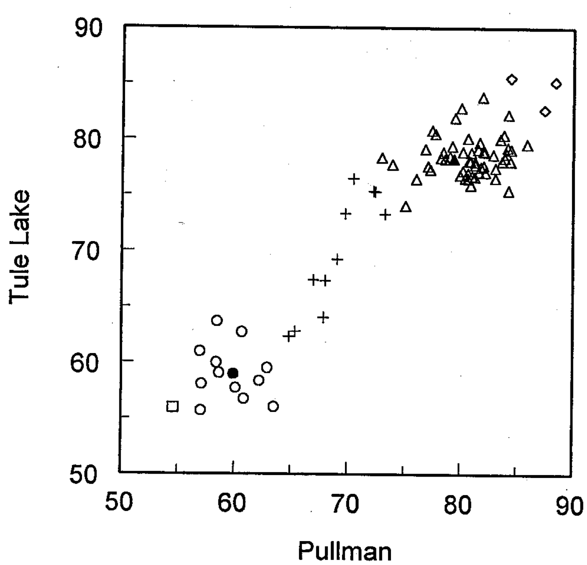

Fig. 5. Single-kernel characterization system hardness of 83 homozygous chromosome 5D recombinant substitution wheat lines derived from Chinese Spring (CS) and substitution line Chinese Spring (Cheyenne 5D) (CS(CNN5D)) grown at Pullman, WA, and Tule Lake, CA. Parents and recombinant lines are denoted as: CS: ●; CS(CNN5D): ▲; lines not significantly different from CS: ○; lines not significantly different from CS(CNN5D): △; lines significantly harder than CS(CNN5D): ◇; lines significantly softer than CS: □; and lines significantly different from and in between the two parental classes: +. Duncan's multiple range test parental class limits were 55.3–61.7 for CS and 74.6–83.2 for CS(CNN5D).

The level of mechanical starch damage present in break flour obtained from Tule Lake samples was highly correlated with grain hardness. Correlation coefficients for percent starch damage and NIR and SKCS hardness were both $r = 0.78$ and for percent bran and midds were $r = –0.71$ and 0.71, respectively. Starch damage and break flour yield were not correlated (data not shown).

DISCUSSION

All methods of measuring wheat grain hardness, except the fundamental engineering approach of Glen et al (1991), are empirical and subject to definition. All, however, strive to reflect the fundamental differences in endosperm material properties conferred by the hardness locus, *Ha*, on the short arm of chromosome 5D (Mattern et al 1973, Law et al 1978) and probably other minor loci. Our results support the segregation of one major hardness locus with allelic differences between Chinese Spring and Cheyenne. The results also support prior reports of additional minor genes action (Symes 1965, Baker 1977), with some evidence of transgressive segregation.

The classification of recombinant lines according to parental values, using DMRT (Figs. 1–5), indicates that recombination of minor gene(s) produces transgressive segregation for lines harder than the hard parent (Quadrumat milling midds, NIR, and SKCS) and softer than the soft parent (SKCS) and greater percent bran yield than Chinese Spring. The three best (most discriminating) methods of measuring grain hardness—Quadrumat bran percentage, NIR, and SKCS—all identified a significant number of recombinant lines (14–25, whole ANOVA model) as being different from either parent but in between the parental classes. The presence of this intermediate class is a further indication of the effect of minor gene(s) on the action or presence of the two allelic forms of *Ha*. Indeed, Giroux and Morris (1997) showed that the lines with similar hardness to Chinese Spring and most of the lines with intermediate hardness contained the same allelic form of puroindoline b (*Pinb-D1a*). Clearly, segregation of other genes on chromosome 5D (days to heading and the *Vrn3/vrn3* locus on the long arm of 5D) were present in this population. The apparent distortion of an expected ratio of 1:1 for these traits and hardness traits may support the presence of other genes on chromosome 5D or may be the result of factors that affected the transmission rate of gametes during the creation of the lines.

Expression of grain hardness is highly influenced by the environment under which plants grow and mature, although genotypes responded similarly, as evidenced by low line-location ANOVA interaction terms (Table II) and high correlations between two dissimilar locations (Figs. 1–5). Although the various measures of grain hardness were highly intercorrelated, they each revealed subtle differences in grain properties and the way in which the properties segregate between the two parents. Supposedly, these methods reflect the underlying physical principles of kernel fracture, although methods such as Quadrumat milling also may be influenced by such traits as bran thickness and kernel morphology. Of the grain traits measured (test weight, protein content, kernel weight and size), none was well correlated with hardness. The poor correlations indicate that traits such as protein and kernel size are relatively independent of grain hardness, and the measures of grain hardness

TABLE III
Correlations (r) Among Methods of Measuring Grain Hardness in Wheat[a]

Method[b]	Quadrumat Milling Fraction: Bran	Quadrumat Milling Fraction: Middlings	Quadrumat Milling Fraction: Break Flour	NIR Hardness
Quadrumat				
Middlings (%)	–0.67***	…	…	…
Break flour (%)	–0.11*	–0.66***	…	…
NIR	–0.85***	0.71***	–0.08 ns	…
SKCS	–0.78***	0.75***	–0.22***	0.87***

[a] ns, *, and *** indicate $P > 0.05$, = 0.01–0.05, and < 0.001, respectively.
[b] NIR = near infrared reflectance; SKCS = single-kernel characterization system.

are not inadvertently confounded with other grain traits (Yamazaki and Donelson 1983, Pomeranz and Williams 1990).

Generally, the different methods of measuring grain hardness were well correlated (Table III). Due to its ability to provide the greatest differentiation between the two parents and among the recombinant lines (Tables II; Fig. 5), SKCS was judged the best method of measuring wheat grain hardness. Additionally, the SKCS instrument is easy to operate and insensitive to a range of operating conditions (Osborne et al 1997). A further advantage of the SKCS instrument is the additional kernel data it provides (Martin et al 1993, Osborne et al 1997).

The results obtained for break flour yield (Fig. 4) were unexpected, in that higher break flour yield traditionally is associated with softer grain (Yamazaki and Donelson 1983, Finney and Andrews 1986) and serves as one of the primary assessments of soft wheat quality in the United States (P. L. Finney and C. S. Gaines, *personal communication*). In the current study, the two parents differed little in percent break flour, and the DMRT parental classes nearly completely overlapped. A key difference between the Quadrumat milling in our study and routine milling procedures is the manner in which grain moisture content is increased by a procedure referred to as "tempering." In routine milling, water is added to grain that is then held from several hours to <2 days. In the current study, no tempering water was added; rather, grain moisture content was raised by increasing the equilibrium moisture content in an elevated relative humidity environment. It seems quite likely that an initial excess of tempering water could rearrange cellular constituents in the endosperm, unlike water in the gaseous state.

Clearly, the results of Bechtel et al (1996) point to a role played by water and changes that occur during dehydration, such that events during rehydration may be important. Finney and Andrews (1986) found that tempering grain dramatically improved correlations between break flour yield and particle size index, both for a set of soft wheats (r = 0.46 improved to 0.87) and a mixed set of soft and hard wheats (r = 0.70 improved to 0.92). The more general relationship between moisture content and measured grain hardness is well documented (Yamazaki and Donelson 1983, Finney and Andrews 1986, Pomeranz and Williams 1990) and shows an increase in softness with increasing moisture content. The second surprising result in regard to break flour yield is that a significant number of lines, 18 in the combined ANOVA, yielded significantly less break flour than either parent (Fig. 3); this result may be the most compelling suggestion that minor genes from both parents recombine to produce lower break flour types.

The primary determinant of wheat grain hardness appears to be related to factors that determine the adhesion of endosperm components (Stenvert and Kingswood 1977). Greater adhesion of the starch granule-protein matrix results in increased starch damage among hard wheats. We also found that the level of starch damage of Quadrumat milled break flours was well correlated with the NIR and SKCS indexes (both r = 0.78). In a companion article (Giroux and Morris 1997), we described a change in one of the two puroindoline proteins coded by the *Ha* locus that ultimately may explain the greater adhesion of endosperm cellular components of hard wheat.

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