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A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin

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Abstract The quantitative level of friabilin 15-kDa protein present on the surface of water-washed starch is highly correlated with wheat grain softness. Friabilin is composed primarily, if not exclusively, of the proteins puroindoline a and b. The transcript levels of these two proteins are similar among hard and soft wheat varieties, and the expression of both is controlled by the short arm of chromosome 5D, also the chromosomal location of the *Hardness* gene. We report here a glycine to serine sequence change in puroindoline b associated with hard grain texture. This amino acid change results from a single nucleotide mutation and resides in a region thought to be important for the lipid-binding properties of puroindolines. No recombination was observed between the serine puroindoline-b mutation, hard grain texture and low levels of starch surface friabilin among a set of 83 homozygous 5D recombinant lines derived from the soft-textured variety 'Chinese Spring' and the substitution line 'Chinese Spring' containing the 5D chromosome of the hard-textured variety 'Cheyenne'. The sequence change reported here may adversely affect the lipid-binding properties of puroindoline-b and so effect hard grain texture. The results suggest that grain hardness results from puroindoline-b functionality such that the *Hardness* gene is a direct manifestation of puroindoline structure. We are suggesting the tentative molecular marker loci designations of *Pinb-D1a* and *Pinb-D1b* for the glycine and serine puroindoline-b types, respectively.

Key words *Triticum aestivum* L · Puroindoline · Friabilin · Wheat grain hardness

Abbreviations *BSA* Bovine serum albumin · *LMW* low molecular weight · *TX114* Triton X-114 · *GPI* glycosyl phosphatidylinositol · *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis · *TCA* trichloroacetic acid · *SKCS* Single Kernel Characterization System · *DAF* days after flowering · *NIR* near-infrared reflectance · *RT-PCR* reverse-transcription polymerase chain reaction · *EDTA* ethylenediaminetetra-acetate · *MOPS* 3-[*N*-morpholino]propanesulfonic acid

Introduction

The varieties of cultivated wheat (*Triticum aestivum* L. and *T. durum* Desf.) can be separated into three distinct classes of grain hardness: soft hexaploid, hard hexaploid and durum. Grain hardness has a profound effect on the milling, baking and end-use quality of wheat. In the context of wheat grain, hardness or more appropriately *texture* is normally defined as (1) the resistance to deformation or fracture properties, (2) the particle size distribution after grinding or milling or (3) the level of starch damage after grinding or milling (Pomeranz and Williams 1990; Anjum and Walker 1991; MacRitchie 1980; Glenn et al. 1991). Soft wheat kernels fracture more easily, release numerous intact starch granules and produce finer-textured flours with less starch damage. Hard wheats produce coarser-textured flours and exhibit fracture planes that produce broken starch granules, hence the higher levels of starch

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damage. Durum wheats represent an additional increase in grain hardness with greater force required to fracture, even coarser-textured meals after grinding or milling, and the greatest level of damaged starch.

Kernel hardness is inherited simply and is controlled by one or two major genes and perhaps one or more minor genes (Symes 1965; Baker 1977; Anjum and Walker 1991). The major gene that controls grain hardness, *Ha*, is located on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). As such, there exist the two allelic forms in hexaploid wheat, whereas durum is null.

Differences in hardness may involve the adhesion of starch and matrix protein or the continuity of the protein matrix and the strength with which it physically entraps starch granules (Barlow et al. 1973; Simmonds et al. 1973; Stenvert and Kingswood 1977). Greenwell and Schofield (1986) reported the existence of a 15-kDa protein, termed friabilin, that was abundant on the surface of water-washed soft wheat starch, scarce on hard wheat starch and absent on durum wheat starch. This correlation between the qualitative level of friabilin and grain hardness has remained unbroken among hundreds of wheats from around the world (Bettge et al. 1995 and references therein).

Friabilin is composed of multiple forms of closely related proteins found on water-washed wheat starch (Morris et al. 1994). Results of Giroux and Morris (personal communication) indicate that the two main components of friabilin are the proteins puroindoline a and b and that these are probably GPI-linked membrane proteins. Puroindoline a and b have been cloned and sequenced and found to be rich in tryptophan (4.2 mol% and 5.0 mol%, respectively) (Gautier et al. 1994). Puroindolines are also soluble in TX114 (Blochet et al. 1993). The high tryptophan content and TX114 solubility are both characteristics of integral membrane proteins (Bordier 1981; Schiffer et al. 1992). Marion et al. (1994) demonstrated strong interactions between puroindolines and polar lipids in synthetic membrane bilayers and suggested that the tryptophan-rich domains of puroindoline a and b form membrane-anchoring loops between α-helices. These lipid-binding properties of the puroindolines likely relate to both the presence of a markedly greater quantity of bound polar lipids (glyco- and phospholipids) on the surface of water-washed starch from soft wheats than from hard wheats and that bound polar lipids appear to be involved in the interaction of puroindolines and the granule surface (Greenblatt et al. 1995). Both puroindolines and bound polar lipids are absent from water-washed durum starch.

We present here data on a single nucleotide change in the sequence of puroindoline b that confers a glycine to serine change in the expressed protein. The sequence difference resides near the tryptophan-rich domain and would likely change the secondary or tertiary structure, reducing both the hydrophobicity and strength of the lipid binding of puroindoline b. Among a genetically defined set of 83 hard/soft homozygous chromosome 5D recombinant substitution lines, no recombination was detected between the serine sequence change, grain hardness and low levels of starch-associated friabilin. We speculate that this sequence change, which is also consistent in two other unrelated hard and soft hexaploid wheat varieties, may change the affinity of puroindoline b for lipids such that hard grain texture results.

Materials and methods

Plant culture and measurement of grain hardness

The 83 homozygous 5D recombinant substitution lines were developed from the soft red wheat 'Chinese Spring' and the hard red winter wheat cultivar 'Cheyenne' by R. S. Kota and J. Dvorak at the University of California-Davis following the procedure of Law (1966). Each line contains 20 pairs of normal euploid 'Chinese Spring' (CS) and 1 pair of recombined Chinese Spring/Cheyenne 5D chromosomes [CS(CNN5D)]. 'Langdon' durum, the 'Langdon' durum substitution line CS5D(5B), and CS Nulli 5D/Tetra 5A were grown in a greenhouse using common cultural practices.

Grain hardness was measured using a near-infrared spectrometer (model 450, Technicon, Tarrytown, N.Y.) on UDY ground grain (Method 39-70A; AACC 1995). Single kernel hardness readings were obtained by analyzing 300-kernel samples of grain for hardness, moisture and kernel size and weight using the Perten Model SKCS 4100 following the manufacturer's suggested operating procedure (Perten Instruments, Reno, Nev.). Single Kernel Characterization System (SKCS) and Near-Infrared Reflectance (NIR) grain hardness readings represent the mean of two replications grown at two locations.

Starch-surface protein isolation and SDS-PAGE

Grain was produced in replicated field plots at the Spillman Agricultural Farm, Pullman, Wash., in 1994 and milled into flour on a Quadrumat mill. Data presented on friabilin levels represent the mean of two to four individual measurements obtained as follows.

Starch granule surface proteins were isolated from 100 mg of flour by a modified single-kernel procedure (Bettge et al. 1995) and electrophoretically separated using SDS-PAGE. SDS-PAGE was performed using standard methods (Laemmli 1970) using 13.5% T, 2.6% C and 0.75-mm thick minigels (Bio-Rad). Standard 10-well gels were run for 55 min at 200 V and silver-stained by a TCA fixation method (Morris et al. 1994). Each sample was visually rated for the presence of friabilin at 15 kDa. Additionally, the amount of friabilin present in the 15-kDa region was quantified by scanning black and white negatives of silver stained gels with a Pulnix TM-7CM video camera interfaced with NIH Image 1.5 software. The friabilin band was quantified by integrating the densitometric scan for the 15-kDa band for each individual lane. Bands staining in the 65- to 70-kDa molecular-weight region were quantified for each lane and used to correct loading discrepancies. Values were plotted on standard curves generated using a dilution series of the soft CS parent.

Northern blot analysis

RNA was isolated by a LiCl method (McCarty 1986) from 10–14 DAF developing kernels that had been quick frozen in liquid N_2.

RNA samples were denatured in 2.2 *M* formaldehyde/50% formamide and fractionated on 1% agarose/2.2 *M* formaldehyde gels using a MOPS buffer (Maniatis et al. 1982). Gels were blotted onto nylon membrane (Hybond N, Amersham) using 10 × SSPE [1.8 *M* NaCl, 100 m*M* Na_2HPO_4 (pH 7.7) and 10 m*M* EDTA] and crosslinked to the wet membrane by 6 min of UV irradiation (3 J/cm^2).

Hybridizations were performed in 0.5 *M* Na_2HPO_4 (pH 7.2), 7% (w/v) SDS and 1% (w/v) BSA (Church and Gilbert 1984). Blots were pre-hybridized for 1 h at 67°C and hybridized for 18 h at 67°C. Puroindoline-a and -b probes were prepared by the random primer method (Gibco/BRL, Life Technologies, Gaithersburg, Md.) to a specific activity of greater than 1×10^9 cpm/μg DNA. Blots were washed twice in 2 × SSPE, 0.1% SDS and twice with 0.2 × SSPE, 0.1% SDS, each wash at 67°C for 30 min.

DNA isolation and PCR amplification of puroindoline a and b

High-molecular-weight DNA was isolated by the procedure of Dellaporta et al. (1983). Amplification of puroindoline a and b was performed using puroindoline a- and b-specific primers (Gautier et al. 1994). RT-PCR was performed on RNA samples that had been treated with DNAse (Amplification grade, BRL) using oligo-dT and standard procedures (BRL). No products were obtained in control reactions lacking added reverse transcriptase. Specific PCR products were purified from primers and dNTPs with disposable filter units (30,000 NMWL cut-off, Millipore) and sequenced directly using the amplification primers.

Amplification of puroindoline b from each of the 83 homozygous recombinant substitution lines and parents was conducted using 0.2 μg of genomic DNA. A fraction of each PCR reaction was electrophoresed on 1.5% (w/v) agarose gels. The 3′ primer specific for the CS puroindoline b sequence type was the reverse complement of 5′GGCGGCTGTGAGCATGAG 3′. The 3′ primer specific for the CNN puroindoline-b sequence type was the reverse complement of 5′AGCGGCTGTGAGCATGAG 3′.

Sequence comparisons were performed using the GCG program (Genetics Computer Group, Madison, Wis.). Structural predictions were made using the GCG programs that utilizes the model of Garnier et al. (1978) and the algorithm of Jameson and Wolf (1988).

Results

Puroindoline-a and -b transcript levels are similar in hard and soft wheat and controlled by chromosome 5D

Friabilin is composed primarily, if not exclusively, of the tryptophan-rich proteins puroindoline a and b. The transcripts of both of these genes are present in soft and hard recombinants derived from the soft-textured CS and the hard-textured substitution line CS(CNN5D) (Fig. 1). Additionally, puroindoline-a and -b transcript levels were similar in the hard red winter cultivar 'Wanser' (Fig. 1) and the soft white winter wheat cultivar 'Hill 81' (data not shown).

Levels of both transcripts were controlled by chromosome 5D as evidenced by the lack of puroindoline transcripts in CS Nulli 5D/Tetra 5A (Fig. 1). Additionally, no transcripts were detected in the durum cultivar 'Langdon'. The substitution of CS chromosome 5D for Langdon 5B [LDN 5D(5B)] restored both puroindoline-a and -b transcripts and grain softness. The SKCS hardness of 'Langdon' was 88, whereas that of the LDN 5D(5B) substitution line was 24. Transcript levels of actin, a control gene, were roughly equal in each of the genotypes (data not shown).

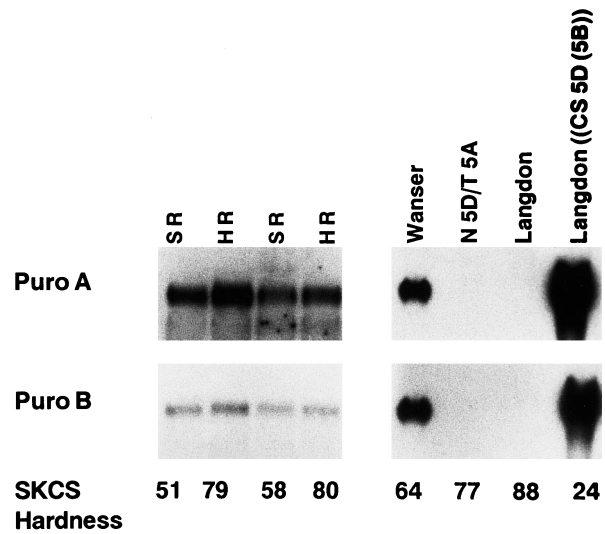


Fig. 1 Example of Northern blot analysis of puroindoline-a (*Puro A*) and -b (*Puro B*) transcript levels among soft and hard recombinants of 'Chinese Spring' and the substitution line Chinese Spring (Cheyenne 5D) at 10 days after flowering (DAF) (*left two panels*). *SR* and *HR* denote soft and hard recombinants, respectively. Each lane was loaded with 10 μg of total RNA. The *two rightmost panels* represent puroindoline a- and b-probed blots prepared using 10 μg of RNA per lane from developing heads collected at 14 DAF from the hard red winter wheat cultivar 'Wanser', 'Chinese Spring' Nullisomic 5D/Tetrasomic 5A (N5D/T5A), 'Langdon' durum, and the 'Langdon' substitution line Langdon ((CS5D(5B)) where the pair of 5B chromosomes of 'Langdon' were replaced by the 5D of 'Chinese Spring'. SKCS hardness denotes grain hardness as determined by the Perten 4100 Single Kernel Characterization System

A glycine to serine change in puroindoline b is associated with hard grain texture

Puroindoline-a and -b transcripts from the soft-textured varieties CS and 'Hill 81' and the hard-textured varieties 'Cheyenne' and 'Wanser' were sequenced. No changes were found in the coding sequences of puroindoline a among the four genotypes relative to that reported by Gautier et al. (1994) (as submitted to GenBank) (data not shown). However, a single nucleotide change was found in the coding sequence of puroindoline b for both of the hard-textured varieties near the trailing end of the tryptophan-rich domain (Fig. 2). Both of these hard wheat varieties contained a DNA base change which results in a glycine to serine amino acid change (position 46). In the soft varieties, the two glycine residues adjacent to the tryptophan-rich domain are conserved both within the sequences of puroindoline a and b as well as in the wheat 9-kDa lipid transfer protein (Marion et al. 1994).

Position	39	40	41	42	43	44	45	46	47	48	49
Chinese Spring (soft)	TGG W	CCC P	ACA T	AAA K	TGG W	TGG W	AAG K	GGC G	GGC G	TGT C	GAG E
CS(CNN5D) (hard)	TGG W	CCC P	ACA T	AAA K	TGG W	TGG W	AAG K	**AGC** **S**	GGC G	TGT C	GAG E
Hill 81 (soft)	TGG W	CCC P	ACA T	AAA K	TGG W	TGG W	AAG K	GGC G	GGC G	TGT C	GAG E
Wanser (hard)	TGG W	CCC P	ACA T	AAA K	TGG W	TGG W	AAG K	**AGC** **S**	GGC G	TGT C	GAG E

Fig. 2 DNA and deduced amino acid sequence of the tryptophan-rich domain of puroindoline b isolated via RT-PCR from 10 DAF RNA from 'Chinese Spring' (soft), the substitution line Chinese Spring (Cheyenne 5D) (hard), 'Hill 81' (soft) and 'Wanser' (hard). The guanine to adenine base change and the resultant glycine to serine amino acid change at position 46 are *underlined* and in *bold*. No other amino acid sequence changes were found in the remainder of puroindoline b or in all of puroindoline a in comparison to that reported by Gautier et al. (1994) (as submitted to Genbank)

No recombination observed between puroindoline-b mutation and grain hardness

A set of 83 homozygous recombinant 5D substitution lines derived from the soft variety CS and the hard CS(CNN5D) substitution line were examined for recombination between the puroindoline-b glycine to serine sequence change (present on chromosome 5D of the hard cultivar CNN) and hard grain texture. The glycine to serine change was detected using a PCR test performed on genomic DNA using 3′ primers that were specific to either the glycine or serine codon. Expected size of the PCR products with both primers is approximately 250 bp. Figure 3 shows an example of PCR analysis of hard and soft recombinants with the CS (glycine soft type) puroindoline b-specific DNA primer.

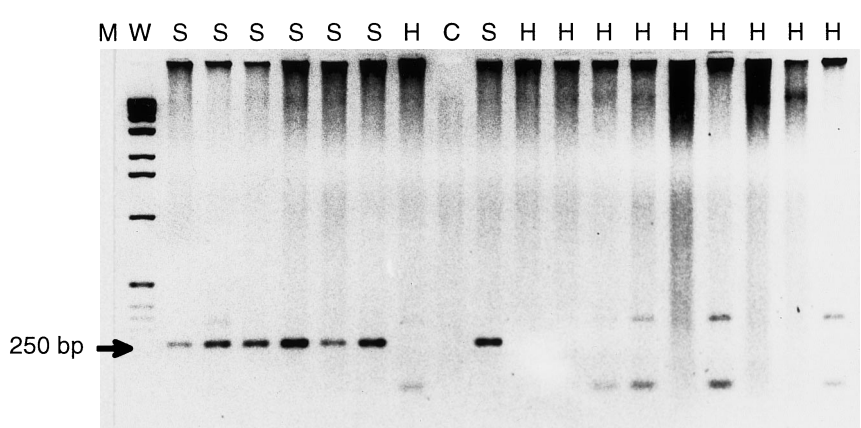
Fig. 3 Negative image of a fluorescent ethidium bromide-stained gel of PCR products obtained using a glycine codon-specific PCR primer derived from the puroindoline b sequence of 'Chinese Spring'. The reaction used genomic DNA from chromosome 5D hard/soft recombinants. *S* and *H* indicate soft and hard recombinants, respectively, as determined by Morris, DeMacon and Giroux (data not shown). Lane marked *C* designates a control PCR performed in the absence of added genomic DNA

Each recombinant line produced only one of the two possible PCR products, i.e. only the CS glycine or the CS(CNN5D) serine type. PCR products smaller and larger than the expected size of 250 bp were inconsistent and of unknown origin. The lanes shown in Fig. 3 have been labeled according to the hardness class as determined by Morris, DeMacon and Giroux (personal communication).

The frequency distribution of grain hardness (SKCS single kernel measure) of the 83 recombinant lines indicates the likely segregation of only one major genetic component (Fig. 4A). One group was tightly clustered around a grain hardness of approximately 78. The frequency of hardness in the softer portion of the range (i.e. <73) was more disperse with a second peak at 58. NIR hardness readings provided essentially the same distribution (data not shown).

A plot of grain hardness using both NIR and SKCS hardness readings with the puroindoline-b sequence type (glycine or serine) highlighted that there was no recombination between puroindoline b and grain hardness (Fig. 4B). However, the two classes are nearly continuous, and no significant gap exists between them. This continuous distribution and the greater spread of hardness readings among the soft (glycine-type) recombinants is possibly due to either segregation or epistasis of additional minor genes that influence hardness. Few soft recombinants had NIR and SKCS hardness readings as soft as the original soft CS parent. It should be noted that the measurement of phenotypic hardness has inherent variation (Morris, DeMacon and Giroux, data not shown).

Friabilin, which is comprised of puroindoline a and b (Giroux and Morris, data not shown), serves as a marker of grain hardness based on its relative abundance on the surface of water-washed starch. The expression of this trait is controlled by a gene, possibly the *Ha* gene itself, on 5DS. Additionally, the occurrence of friabilin exhibits additive inheritance in the triploid endosperm (Bettge et al. 1995). Here, the levels of friabilin present on the surface of starch were quantified by measuring the intensity of stained friabilin bands in



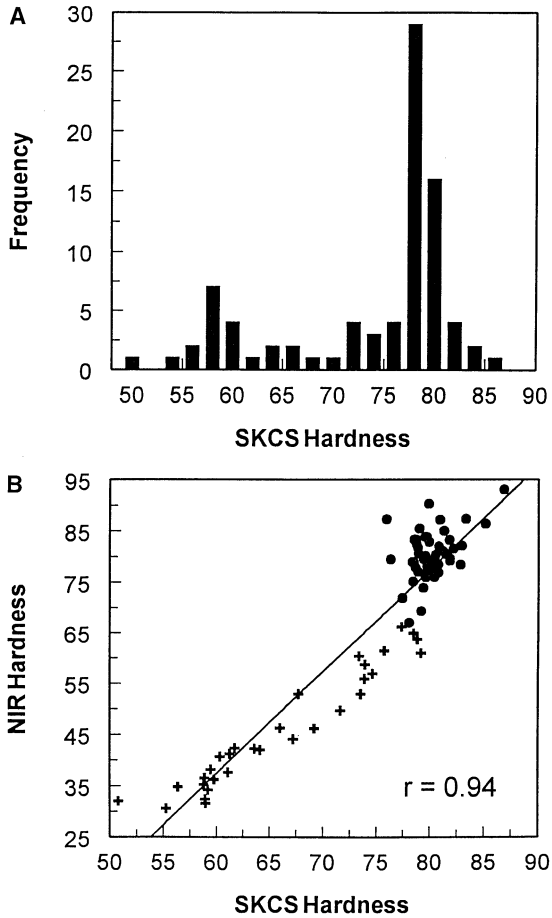


Fig. 4 **A** Frequency distribution histogram of SKCS single-kernel hardness readings of the 83 hard/soft chromosome 5D recombinants and parents, 'Chinese Spring' and 'Chinese Spring' substituted Cheyenne 5D. Readings are the average of two replications for each of two locations. Parental values were 60 and 79, CS and CS(CNN5D), respectively. **B** SKCS Single Kernel versus NIR hardness readings. Recombinants are classified according to puroindoline-b sequence type where (+) denotes the glycine sequence type of the soft parent, 'Chinese Spring', and (●) denotes the serine sequence type of the hard parent 'Chinese Spring' substituted Cheyenne 5D

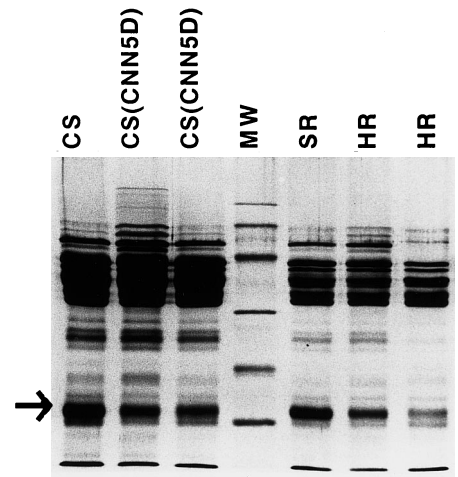


Fig. 5 Gel photo of starch surface proteins prepared from the soft parent 'Chinese Spring' (*CS*), the hard parent 'Chinese Spring' substituted Cheyenne 5D (*CS*(*CNN5D*)) (two replicates), and one soft and two hard recombinants (*SR* and *HR*, respectively). Molecular weight markers (*MW*) are 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. *Arrow* indicates 15-kDa friabilin

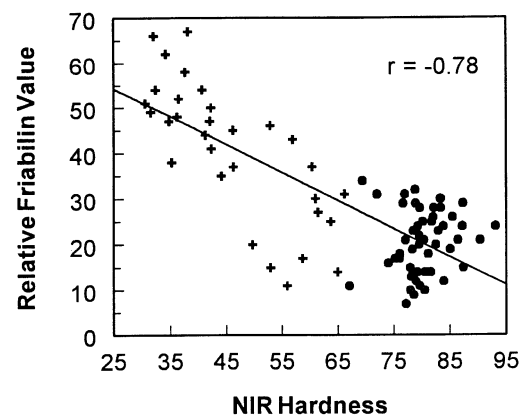


Fig. 6 Relative amount of starch surface friabilin as a percentage of the soft parent 'Chinese Spring' for soft/hard recombinants versus NIR hardness. Recombinants are classified according to puroindoline-b sequence type where (+) denotes the glycine sequence type of the soft parent, 'Chinese Spring', and (●) denotes the serine sequence type of the hard parent 'Chinese Spring' substituted Cheyenne 5D

SDS-PAGE and expressed as a percentage relative to the CS parent (Fig. 5). The relative friabilin values of each of the recombinant lines and their NIR hardness readings are shown in Fig. 6. No recombinant had as much starch surface friabilin as the soft parent CS. The correlation between starch surface friabilin and grain hardness was $r = -0.78$. Again, adding to the plot a notation of the PCR results for puroindoline b, i.e. glycine or serine, indicates that no recombination occurred between low levels of starch surface friabilin, hard grain texture and the puroindoline-b serine sequence change. The overall distribution of the recombinant lines was generally similar to that seen in Fig. 4B.

Discussion

Wheat grain hardness is relatively simply inherited (Symes 1965; Baker 1977; Anjum and Walker 1991), and most of the variation among the classes soft hexaploid, hard hexaploid and durum can be assigned to either the allelic state of a single locus, *Ha*, on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978) (Figs. 1 and 4A) or the absence of the locus in the case of durum. We found that among the chromosome 5D recombinant substitution lines, the level of friabilin was without exception well-correlated with hardness

(Figs. 5 and 6). No evidence was found for recombination; that is, soft recombinant lines having little or no starch surface friabilin. This result, using defined genetic stocks, is consistent with that obtained over the past decade, i.e. no exception to the "high surface friabilin – soft wheat texture" rule has been reported (Bettge et al. 1995; Greenblatt et al. 1995; Morris et al. 1994; Oda et al. 1992). Consequently, the abundance of friabilin on the surface of water washed starch and grain softness (*Ha*) are considered to be either traits encoded by independent but very tightly linked genes or, more probably, pleitropic effects of the same gene.

This dramatic difference in starch surface friabilin appears to be due to a partitioning or affinity phenomenon during starch isolation and not a direct difference in friabilin gene expression between soft and hard wheat (Greenblatt et al. 1995). Jolly et al. (1993) showed that friabilin levels in native endosperm were similar in both soft and hard wheats. We found that, additionally, the transcript levels of the friabilin components puroindoline a and b are similar between hard and soft wheats (Fig. 1). Consequently, a model of grain hardness involving dramatically different levels of a "non-stick" protein (friabilin) present in soft wheat but nearly lacking in hard wheat appears untenable (Schofield and Greenwell, 1987).

One possible resolution to the apparent discrepancy between levels of friabilin/puroindolines found on the surface of water-washed starch as opposed to levels in native endosperm may relate to the interaction of puroindolines with polar lipids. Two classes of bound polar lipids exhibit the same pattern of occurrence as friabilin, i.e. abundant on the surface of water washed soft wheat starch but scarce on hard (Greenblatt et al. 1995). Further, friabilin association with the surface of the starch granule appears to involve these polar lipids (Greenblatt et al. 1995). A second possibility is that friabilin from soft and hard wheat differ such that their affinity for lipids also differs.

Friabilin is not a single, discrete polypeptide, but rather is comprised of the related proteins puroindoline a and b (Giroux and Morris, data not shown). In accordance with the protein data of Jolly et al. (1993), both soft and hard hexaploid wheats exhibit similar levels of puroindoline a and b transcripts; however Chinese Spring nulli 5D/tetra 5A, which lacks chromosome 5D, and the AABB tetraploid durum cultivar, 'Langdon', contained no detectable transcripts of these genes (Fig. 1). The substitution of 'Chinese Spring' chromosome 5D for 5B of 'Langdon' resulted in the expression of puroindoline-a and -b transcripts and soft grain texture in this durum cultivar.

Puroindoline a and b have two glycine residues adjacent to the tryptophan-rich domain (Gly-46 and Gly-47) (Gautier et al. 1994). These two residues are conserved in the 9-kDa lipid transfer protein (LTP) (Marion et al. 1994), in an oat avenin that possesses a tryptophan-rich domain and shares a high degree of homology, and in two unrelated soft wheats reported here (Fig. 2 and data not shown). In two unrelated hard wheats, however, we found a single base change in the DNA sequence of the puroindoline-b gene that effects a change from glycine to serine (Gly-46 to Ser-46) (Fig. 2). As such, we consider the glycine sequence type to be the wild type or non-mutant type. Softness, *HaHa*, is dominant (Mattern et al. 1973; Law et al. 1978) and is inherited additively in the triploid endosperm (Bettge et al. 1995). Recombination between the serine sequence change present in 'Cheyenne' and 'Wanser' and hard grain texture would be expected if puroindolines are unrelated or unlinked to the Hardness gene. We found no such recombination between the sequence type of 'Cheyenne' and 'Chinese Spring' among 83 homozygous 5D recombinant substitution lines (Fig. 4B). If linkage is assumed to be involved, the maximum map distance at a 95% confidence level would be 4.28 cM (Table 14A in Steel et al. 1997).

The segregation of additional minor genes is indeed a possibility among the soft recombinants. In fact, Jolly et al. (1996) found that some GSP-1 clones detected restriction fragment length polymorphisms (RFLPs) that were linked to *Ha*. These and other related genes may represent minor hardness differences among our CS sequence type recombinants.

We are suggesting the tentative molecular marker designations *Pina-D1*, for puroindoline a, and *Pinb-D1a* and *Pinb-D1b*, for the puroindoline-b glycine- and serine-type sequences, respectively. These designations for the wild type follow the revised Guidelines for Nomenclature of Biochemical/Molecular Loci in Wheat and Related Species (McIntosh et al. 1995); CS provides the prototype allele for *Pinb-D1a*. We have not detected puroindoline proteins or transcripts in durums (AABB) (Fig. 1 and data not shown). However, genomic DNA and protein data on the progenitor species of wheat (Morrison et al. 1992 and data not shown) indicate the existence of a homoeologous series which would tentatively be assigned to the A, B and R genomic loci of puroindolines a and b. The A- and B-genome genes appear to be silent in durums, which is consistent with their hard grain texture.

We consider compelling the sum of data indcating that puroindolines and grain hardness are somehow directly related in a causal way. As such, we may consider how the observed glycine to serine sequence change may alter the structure-function relationship(s) of puroindoline b. The most intriguing structural feature of puroindolines is their tryptophan-rich domains. It therefore seems reasonable to consider that a mutation near this domain would alter the secondary and tertiary structure of puroindoline b. A measure of the membrane affinities of amino acids in this region of puroindoline a and b is shown in Table 1 with individual amino acid affinities as reported by Thorgeirsson et al. (1996). Also included are the

Table 1 Membrane affinity[a] of the individual amino acids comprising or flanking the tryptophan-rich domain of puroindoline b, showing the soft (wild type, Gly-46) and the hard (mutant type, Ser-46) sequences along with their predicted secondary structures according to Jameson and Wolf (1988) and Garnier et al. (1978)[b]

Position	Puroindoline b, soft (*Pinb-D1a*)			Puroindoline b, hard (*Pinb-D1b*)		
	Amino acid	Membrane affinity	Predicted secondary structure[b]	Amino acid	Membrane affinity	Predicted secondary structure[b]
34	D	–	h	D	–	h
35	F	2.40	h	F	2.40	h
36	P	−0.76	–	P	−0.76	–
37	V	1.72	B	V	1.72	B
38	T	0.05	B	T	0.05	B
39	W	2.46	B	W	2.46	B
40	P	−0.76	B	P	−0.76	B
41	T	0.05	B	T	0.05	B
42	K	+	B	K	+	B
43	W	2.46	B	W	2.46	B
44	W	2.46	B	W	2.46	B
45	K	+	T	K	+	T
46	G	0	T	**S**	**−0.26**	T
47	G	0	t	G	0	**T**
48	C	0	H	C	0	**t**
49	E	–	H	E	–	H

[a] According to Thorgeirsson et al. (1996). Positive numbers represent membrane affinity and negative numbers polar affinity. Tryptophan has the greatest membrane affinity at 2.46. Differences between the two puroindoline b types are highlighted

[b] Predicted secondary structure where h = weak helical, H = strong helical, B = strong beta sheet, t = weak turn and T = strong turn. Differences are highlighted

predicted secondary structures (Garnier et al. 1978; Jameson and Wolf 1988).

The tryptophan-rich domain of puroindoline b is predicted to be in a beta sheet conformation and joins two α-helices. The change from a Gly-46 to Ser-46 occurs in a turn of the polypeptide. The change from a glycine to serine also effects a change from no membrane affinity to slightly negative affinity (Thorgeirsson et al. 1996). We conclude that the glycine to serine change may reduce the strength of the lipid binding of this form of puroindoline b. The Gly-46 residue may play an important role in determining the structure/function of puroindoline b. Puroindoline a and b and the 9-kDa lipid transfer protein from wheat share a conserved cysteine backbone (Marion et al. 1994) as well as Gly-46. It is interesting to note that the same serine sequence change was present in two unrelated hard wheat genotypes. Preliminary data indicate that the Ser-46 change is quite common among hard wheats but absent among soft ones (which to date all contain the Gly-46 sequence; data not shown).

In summary, the unbroken linkage between a mutation in the sequence of one of the two friabilin components, puroindoline b, and grain hardness demonstrates that *ha* likely results from a specific structural change in this protein. Differences in the way that soft and hard hexaploid wheat endosperm dries during maturation results in profound differences in the way the grain fractures due to the strength of the starch-protein matrix interface (Bechtel et al. 1996). The possibility that puroindolines affect the way in which amyloplast membranes collapse during desiccation is consistent with localization studies (Dubreil et al. 1994; Giroux and Morris, data not shown) and the presence of higher amounts of membrane structural lipids associated with water-washed starch from soft wheats compared to hard (Greenblatt et al. 1995). We may speculate that puroindoline a and b function in concert to effect grain softness and that a mutation which changes the structure or expression of either protein effects increased grain hardness.

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