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Linkage between RFLP markers and genes affecting kernel hardness in wheat

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Abstract A molecular-marker linkage map of wheat (*Triticum aestivum* L. em. Thell) provides a powerful tool for identifying genomic regions influencing breadmaking quality. A variance analysis for kernel hardness was conducted using 114 recombinant inbred lines (F_7) from a cross between a synthetic and a cultivated wheat. The major gene involved in kernel hardness, *ha* (hard), known to be on chromosome arm 5DS, was found to be closely linked with the locus *Xmta9* corresponding to the gene of puroindoline-a. This locus explained around 63% of the phenotypic variability but there was no evidence that puroindoline-a is the product of *Ha* (soft). Four additional regions located on chromosomes 2A, 2D, 5B, and 6D were shown to have single-factor effects on hardness, while three others situated on chromosomes 5A, 6D and 7A had interaction effects. Positive alleles were contributed by both parents. A three-marker model explains about 75% of the variation for this trait.

Key words Kernel hardness · Wheat · RFLP · QTL · Puroindoline

Introduction

Among the aims for the improvement of cultivated wheat is greater breadmaking quality. Changes in kernel hardness affect many factors important to quality, including milling conditions, granularity, and amount of starch damage (Pomeranz and Williams 1990).

The genetic basis of kernel hardness is relatively well established. Using the particle size index, Worzella (1942) concluded that granularity (a standard measure of hardness) was inherited as a quantitative character but that relatively few genes were concerned. Symes (1965) showed that one major locus was involved. He was able to produce isogenic soft and hard lines for kernel hardness, using the Australian wheat cultivars Falcon and Heron (Symes 1969). Law et al. (1978), studying the genetic control of kernel protein amounts by chromosome 5D, showed that one gene affecting this trait was located close to the major locus influencing hardness, named *ha* (for hardness). This gene was assigned to the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978), but chromosome arms 5AS and 5DL may also carry a minor locus (Morrison et al. 1989). Heritability of the trait is very high, ranging between 0.7 and 0.9 (Williams and Sobering 1984).

The biochemical basis of kernel hardness remains largely unknown. However, because neither storage protein nor starch granule differences could acount for differences in hardness between wheat varieties (Barlow et al. 1973), Simmonds et al. (1973) suggested that hardness is due to a cementing agent between starch and proteins. Hardness was also found to involve the continuity of the protein matrix and the strength with which it physically entrapped starch granules (Stenvert and Kingswood 1977).

More recently, increased amounts of free polar lipids were shown to be strongly correlated with kernel softness (Morrison et al. 1984, 1989). The Mr 15 000 polypeptide, referred to as either "friabilin" (Greenwell and Schofield 1989) or "grain softness protein" (GSP; Jolly et al. 1990, 1993), was found to be a marker of kernel softness. Examination of near-isogenic lines differing in hardness indicated that GSP was associated with *Ha*, suggesting that GSP may be the product of *Ha* and thus the major factor determining the milling characteristics of bread wheats.

Recently, Rahman et al. (1994) showed that GSP is a mixture of different puroindoline-like polypeptides. Puroindolines are basic cysteine-rich proteins (CRP) isolated

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from *Triticum aestivum* (Blochet et al. 1993) which are characterized by a unique tryptophan-rich domain. Characterization of cDNAs showed that puroindolines are synthesized in the form of pre-proproteins and that at least two proteins (puroindoline-a and -b), which are 60% identical, are members of the CRP family (Gautier et al. 1994). Currently no evidence directly implicates puroindolines in softness.

Strategies using either restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) have already been successfully used to map such wheat genes as *Vrn1* and *Fr1* (Galiba et al. 1995) or the leaf rust resistance gene *Lr24* (Schachermayr et al. 1995). In an RFLP mapping study in wheat (Nelson et al. 1995a, b, c) using the population on which the present study is based, we previously identified chromosome arm 5DS markers linked with the kernel-softness gene *Ha*. Here we report the linkage of kernel softness with the puroindoline-a gene, as well as with markers in other regions of the wheat genome.

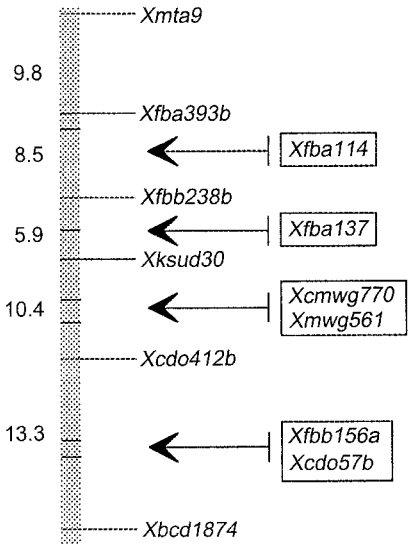

Fig. 1 RFLP map of the wheat chromosome 5D short arm. Markers accompanied by *tick marks* are in LOD 3.0 order. Distances on the left of the chromosome are in Haldane (1919) centiMorgans. Loci in *boxes* were not ordered at LOD 3.0 and are drawn to approximate positions indicated by linkage analysis

Materials and methods

Plant material

The mapping population consisted of 114 SSD lines (F_7 generation) derived from the cross between W-7984 (Synthetic: synthetic amphihexaploid wheat derived from a cross between *T. tauschii* and Altar 84 durum) and Opata 85 (Opata), a hard red spring bread wheat from CIMMYT (Van Deynze et al. 1995) provided by M. Sorrells.

A single row of each entry was sown in the nursery at the INRA station in Clermont-Ferrand, France in 1993 and harvested and analysed in 1994. Plants were grown under normal field conditions with fungicide application to control rusts and powdery mildew. Only 86 of the 114 recombinant inbred lines (RILs) provided enough grain (15 g) for hardness evaluation. Five seeds were also sown in the greenhouse to provide fresh tissue for DNA extraction.

RFLP analysis and mapping

The procedures for RFLP analysis at INRA were performed with digoxigenin as described in Lu et al. (1994) except that alkali-labile DIG-11-dUTP was used to avoid the carryover of signals from probes previously hybridized on the same membrane. RFLP mapping at Cornell was done with radiolabelled probes as described in Nelson et al. (1995b). The restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, and *Dra*I (Boehringer Mannheim) were used for DNA digestion and hybridization following the manufacturer's instructions.

More than 1100 loci were mapped in the population (Nelson et al. 1995a, b, c). Some of the cDNA and genomic DNA probes were described in Van Deynze et al. (1995). We also developed our own library of 500–2000-bp genomic fragments from etiolated seedlings of the varieties Courtot (pTaFBA library) or Chinese Spring (pTaFBB library), cloned in the *Pst*I site of pBluescript vector.

The *mta9* clone described in Gautier et al. (1994) corresponds to the puroindoline-a cDNA obtained from a *T. aestivum* cDNA library constructed with poly(A)+ RNA isolated from immature kernels (23 days after flowering). This clone is 679 bp long and contains a 23-bp 5′-untranslated sequence followed by an uninterrupted reading frame of 444 bp and a 3′- untranslated sequence of 196 bp before the poly(A) tail (16 bp). The primers used for puroindoline-a amplification were 5′- ATGAAGGCCCTCTTCCTCA-3′ (position 43–61 on the sequence) and 5′-TCACCAGTAATAGCCAATAGTG-3′ (position 470–449 on the sequence).

Mapping data were analysed with MAPMAKER version 3.0 (Lander et al. 1987). Loci whose order was established at a LOD of 3.0 were assigned exact positions on the map and the remainder were placed in the intervals in which they best fit using the "place" command. A partial map of chromosome arm 5DS including locus *Xmta9* is given in Figure 1. Complete chromosome maps appear in Van Deynze et al. (1995), Nelson et al. (1995a, b, c), and Marino et al. (1996).

Evaluation of kernel hardness

Kernel hardness was evaluated by near-infrared reflectance spectroscopy (NIR) using an Inframatic 8620 system (Scantec). The analysis followed the methods of AACC (1989) using a Cyclotec lab mill (Tecator) for wholemeal production. Reflectance was measured at 1680 and 2230 nm and hardness was computed with the formula:

$$\text{hardness} = 1475 \times \log(1/R_{2230}) - 1099 \times \log(1/R_{1680}).$$

The NIR instrument is standardized with a batch of five hard samples and five soft samples, with the mean of hard samples made to equal 75 and that of soft samples 25. Owing to genotype/environment interactions, some lines may have scores greater than 100.

Statistical analysis

The associations between markers and kernel hardness were evaluated by a one-way ANOVA using Splus software (Becker et al. 1992). Normality of the residuals was checked with the Pearson test of fitness (Dagnelie 1975). Estimates of the locations of the QTLs and origins of the positive alleles were evaluated using "marker regression" (Kearsey and Hyne 1994) computed with Splus. The additive value of each QTL was estimated via either the least squares estimates (LSmeans) of the general linear model (GLM) procedure SAS^R (SAS Institute Inc. 1991) or marker regression. Interaction effects between loci were also evaluated with the GLM procedure. Interaction parameters between markers M1 and M2 for their respective allelic forms i or j (θ_{ij}) are computed from the LS means estimates as:

$$\theta_{ij} = \text{LSmeans}(ij) - m - a1_i - a2_j$$

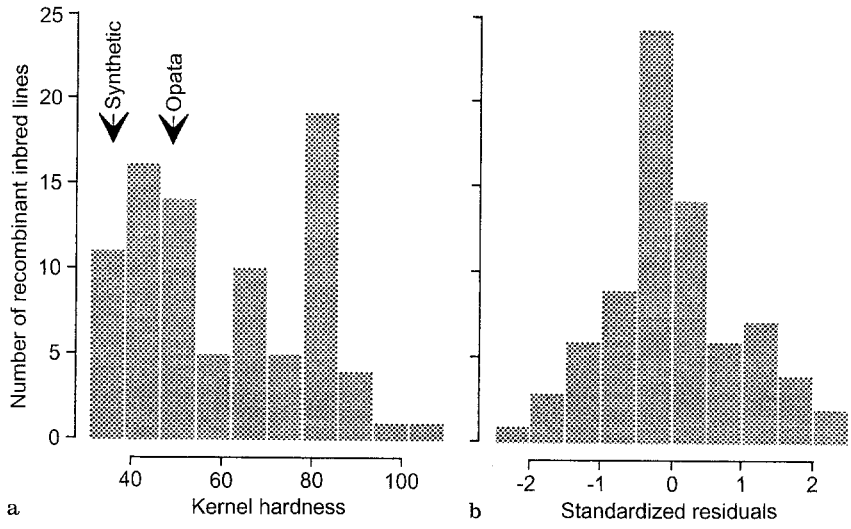

Fig. 2 Distribution of hardness (**a**) and of the standardized residuals for kernel hardness after subtraction of the effect of *Xmta9* (**b**) among the population of recombinant inbred lines

where $a1_i$ and $a2_j$ are the additive effects of markers M1 and M2 with the allelic forms i or j, computed as indicated above, and m is the grand mean for hardness. The significance threshold was set at $\alpha = 0.005$ for main and at 0.001 for interaction effects.

Results

Linkage between molecular markers and kernel hardness

Single-factor analysis

While the two parents of the population do not differ widely in their kernel hardness (Synthetic 35.3; Opata 49.7), the progeny show hardness scores ranging from 31.7 (soft) to 108.2 (very hard) (Fig. 2a). The presence of transgressive lines suggests that both parents possess positive and complementary alleles for this trait.

The shape of the distribution indicates the presence of a major gene controlling hardness which we assume to be *ha*. Coefficients of determination (R^2) found between molecular markers and kernel hardness at $\alpha = 0.005$ are given in Table 1. Locus *Xmta9* on chromosome arm 5DS explains 63.2% of the phenotypic variation of the trait and is obviously linked with *Ha*. Consequently the ANOVA for all other markers but *Xmta9* was performed on *Xmta9* residuals (Fig. 2b) and included main effects for *Xmta9* and M1 and an interaction term between *Xmta9* and M1.

Regions on four other chromosome arms (2AL, 2DL, 5BL, 6DS) each explain 4–6% of the variation (Table 1). The hardness effect from chromosome arms 5DS and 5BL was associated with Opata alleles and that from 2AL, 2DL, and 6DS with Synthetic alleles. This result is consistent with the transgressive segregation for hardness in the RILs despite the similar hardness scores in the parents.

Marker regression and ANOVA detected the same QTLs (Fig. 3). The minimum residual mean square with marker regression occurs at the most-significant locus with ANOVA for chromosome arms 5DS, 2DL and 5BL (Fig. 3A, C, D). On chromosome arm 2AL (Fig. 3B) the minimum is close to *Xfba314* instead of *XksuF11*, probably because *Xfba314* was scored on only 40 RILs. On chromosome arm 6DS (Fig. 3E) the minimum occurs 6 cM from *XksuG48*. This may be due to another locus (*Xbcd1821*) associated with hardness and located 14 cM from *XksuG48*. We suspect that there are two linked QTLs but marker regression will not resolve them closer than about 30 cM apart (Hyne and Kearsey 1995).

Table 1 Markers correlated with kernel hardness at a significance threshold $\alpha = 0.005$. Only the most significant locus of each chromosome arm (Chr.) concerned is indicated. *P*:*F* probability, *df*: degree of freedom: R^2: coefficient of determination of the locus with the main effect (after subtraction of the *Xmta9* effect), Add: additive effect of the locus

Marker	Chr.	*F* value	*P*	*df*	R^2	+allele	Add
XksuF11	2AL	13.99	0.00035	77	0.057	Synthetic	4.63
Xbcd120	2DL	9.99	0.00224	79	0.040	Synthetic	4.95
XksuA1	5BL	14.63	0.00027	76	0.053	Opata	4.56
Xmta9	5DS	142.00	0.00000	83	0.632	Opata	15.11
XksuG48	6DS	11.89	0.00090	80	0.048	Synthetic	3.98

Multiple-factor analysis

Interaction effects between loci showing a significant individual effect and all other loci were analysed to test effects of allelic associations at different loci on kernel hardness. Two models were used: the one described above for interactions between *Xmta9* and other loci, and a three-locus model with effects for *Xmta9*, M1, M2, and M1×M2, where M1 is one of the four loci with a significant main effect and M2 any other locus.

Three interactions were significant at $\alpha = 0.001$ (Table 2). None involves two of the five main loci and each accounts for 4.4–5.1% of the phenotypic variation. In each

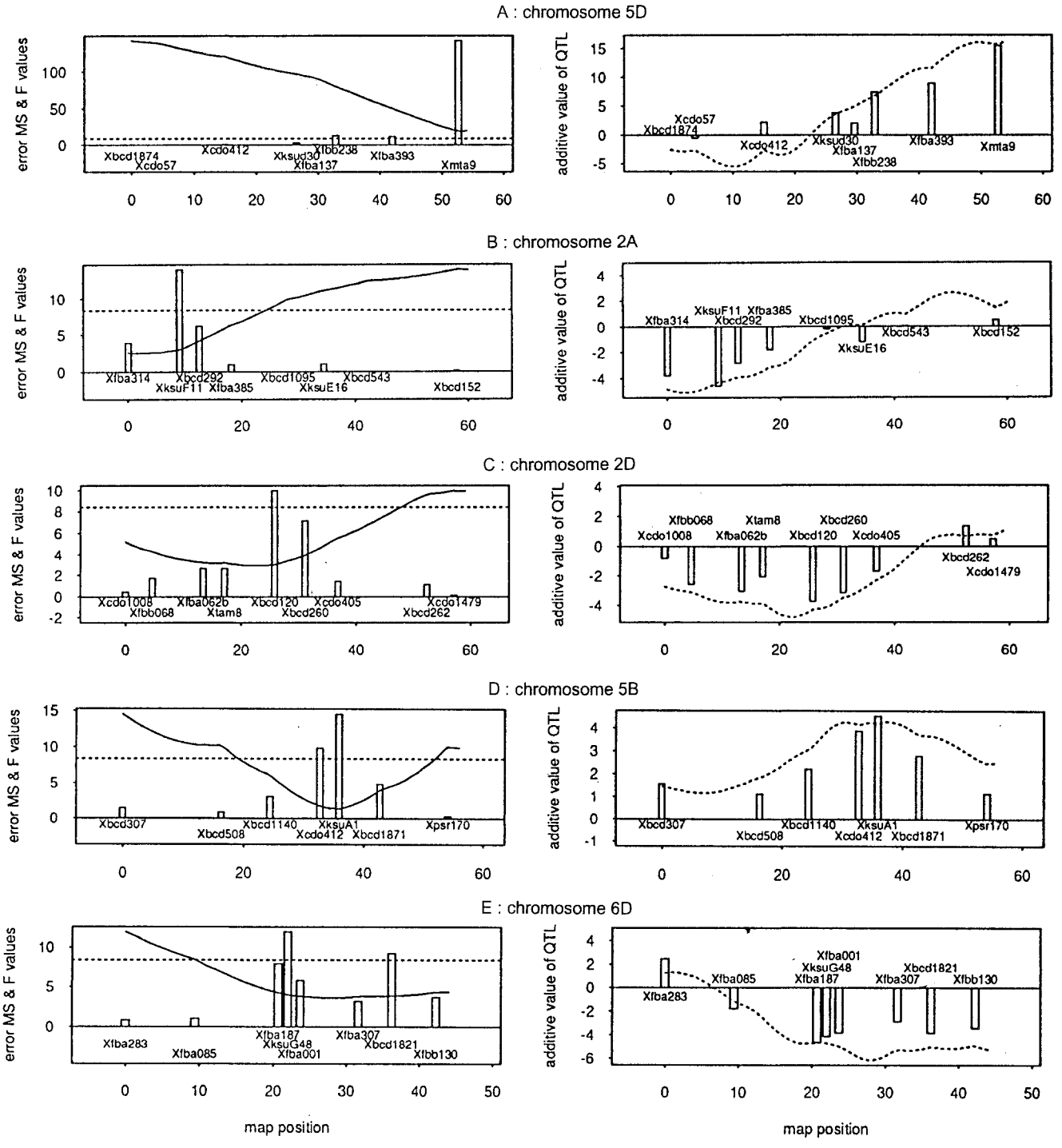

Fig. 3A–E Detection of loci associated with kernel hardness on the RIL population. Only partial maps (around 50 cM long) are given. Positions of the anchor markers are given along the abscissa in Haldane (1919) cM. The long arm of each chromosome is toward the left of the graphics and the short arm toward the right. The origin (*0*) indicates the first marker of the long arm. The graphics on the left represent the F tests for each marker (*vertical bars*). The *dotted line* is located at a *F* value of 8.4 representing a threshold of significance α=0.005 with 80 *df*. The curves indicate the changes in residual mean square for various putative QTL positions as defined by Kearsey and Hyne (1994). The *graphics* on the right represent the differences between the means of the two genotypic classes for each marker locus (m_{Opata}–$m_{Synthetic}$: *vertical bars*). The *dotted lines* correspond to the additive value of the QTL and indicate whether the positive allele (hard) comes from Opata (positive values) or Synthetic (negative values)

case, positive effects appear in coupling phase, when both alleles come from the same parent. The presence of very transgressive plants suggested that complementary alleles would come from different parents (repulsion).

To find the best model explaining hardness in terms of the marker genotypes, we used the STEPWISE procedure from GLM (SAS Institute Inc. 1991). Interactions were not included because of the loss of degrees of freedom due to missing data. The best model includes main effects for loci *Xmta9*, *Xbcd120*, and *XksuF11* and accounts for 74.8% of the total phenotypic variation for kernel hardness. Its robustness was tested with Splus (Becker et al. 1992), using

Table 2 Markers showing significant interactions with kernel hardness at $\alpha = 0.001$. R^2int. is the additional variability explained by the interaction between marker 1 and marker 2. R^2tot. is the total variability explained by the individual effect of markers 1 and 2 and the interaction effect between markers 1 and 2. Coupling means that interactive alleles come from the same parent and repulsion that they come from different parents

Marker 1	Marker 2	Chr.	*P*	R^2int.	R^2tot.	Interaction effects	
						Coupling	Repulsion
XksuA1	*Xcdo457*	5AL	0.00007	0.051	0.123	+3.75	−3.16
Xbcd120	*Xbcd1716*	6DL	0.00023	0.049	0.091	+2.09	−31.2
Xbcd120	*Xcdo475*	7AS	0.00043	0.044	0.091	+2.45	−3.45

re-sampling of 77 recombinant lines with replacement (bootstrap: Efron and Tibshirani 1986). In a set of 300 bootstraps, the main effect of *Xmta9*, *Xbcd120* and *XksuF11* remained significant at $\alpha = 0.01$ in 100%, 95%, and 98% of the sub-samples, respectively. This model explains 12.0 of the 14.4 units of the difference in hardness between the two parents and 46.7 of the 76.5 units of the difference between the individuals with extreme phenotypes.

Discussion

Breeding for hard or soft wheats is of interest for flour quality. Molecular markers tightly linked to genes conditioning softness would enable breeders to predict the phenotype of a new cultivar without waiting for complete homozygosity. Although *Ha* has long been known to be important for kernel softness, we show that this gene cannot explain all of the variation for this trait. Modifying factors increase the variation in softness explained by *Ha* from around 60% to more than 70%.

The closest marker to *Ha* was the gene for puroindoline-a at the end of chromosome arm 5DS. The identity of puroindoline N-terminal sequences with those of friabilin and GSP (Gautier et al. 1994) suggests that these proteins could be the products of *Ha*. However, puroindoline-a has been shown by immunolocalization to lie within and just under the aleurone layer rather than in the central starchy endosperm (Dubreil et al. 1994). Thus, the puroindoline-a gene would be a marker of softness rather than *Ha* itself. This is supported by the plants possessing soft kernels even in the presence of the Opata allele (hard) at the *Xmta9* locus. Moreover, no significant correlation was found between kernel softness and puroindoline-a content in 37 *T. aestivum* cultivars (Dubreil et al. 1994).

Rahman et al. (1994) found that friabilin (or GSP) is a mixture of different puroindoline-like polypeptides. The recent study of Bettge et al. (1995) shows that the occurrence of friabilin on water-washed wheat starch granules is perfectly correlated with grain softness. This might be explained if puroindoline-b, which we found to co-segregate with puroindoline-a at the end of chromosome arm 5DS (data not shown) and which is known to be the main component found on the surface of starch granules (Greenwell cited in Dubreil et al. 1994), is the real product of *Ha*.

Our results are in contrast to those of Nelson et al. (1995c). They assumed that a locus on chromosome 5DS, strongly associated with the percent of vitreous kernels was identical with *ha*, with the *T. tauschii* parent contributing the positive alleles. The literature contains contradictory results on the correlation between vitreousness and hardness; Hong et al. (1989) reported a strong positive correlation, Bakhella et al. (1990) a negative one. The influence of protein on vitreousness could account for the disparate findings. We found no correlation between protein content and hardness in the mapping lines (data not shown). Even if there has been confusion of vitreousness with hardness in the past, they should now be considered to be under the control of different genes genetically linked on chromosome 5DS.

The hybridization pattern of puroindoline-a shows only one fragment in Synthetic and none with Opata (null allele), indicating that there is no puroindoline-a gene in the Opata parent line. This result has been confirmed by a PCR amplification performed on bulks of the ten hardest or softest lines, using specific primers for *mta9* (data not shown). We found no signal for the 'hard' bulk and a clear band with the 'soft' bulk, i.e. the same profile as we observed between Opata and Synthetic. Most wheat probes hybridize to multiples of three fragments indicating loci on homoeologous chromosomes. If there are puroindoline-a genes on chromosomes 5A and 5B, they are insufficiently homologous to be detected by hybridization or PCR.

Other genes are known to map in the same region of the genome and could also be candidate genes for softness. For example, *Fpl2*, a gene contributing to the control of total free polar lipid levels in flour, is also closely linked and might be allelic to *Ha* (Morrison et al. 1989). Some genes coding for lipid transfer proteins (LTPs) are also found on 5D (M. F. Gautier and P. Joudrier, unpublished results), and could also be candidates for *Ha*. Lack of polymorphism between the parental lines for the cloned sequences prevented our mapping them. It also suggests that there are no differences between the allelic forms of Synthetic and Opata, as would be expected if these clones represented *Ha* gene sequences.

Some marker loci found to be involved in kernel hardness mapped in the same regions as storage proteins, namely the gliadin loci on chromosome 6D. Gliadins interact with glutenins in the protein matrix and could conceivably influence hardness via links with glutenins or by their ratio in the total protein content. But no clear genetic link-

age in our study between gliadin loci and hardness supports a biochemical association.

Chromosome arms 5AS and 5DL may also carry minor loci influencing hardness (Law et al. 1978; Morrison et al. 1989). We found no markers in these regions associated with hardness. Perhaps the parental alleles for these putative genes were identical in this cross, or the major 5DS locus, in conjunction with the relatively small population size, may have masked minor effects.

The literature supports the influence of the protein matrix on kernel hardness. It would therefore be interesting to compare the QTL found for hardness with any that may be found for total protein content. Useful validation of the marker associations we report could be provided by testing the same markers on another hardness-characterized population.

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