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Genetic analysis of kernel hardness in bread wheat using PCR-based markers

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Abstract In wheat, kernel hardness is a complex genetic trait involving various directly and indirectly contributing components such as kernel hardness *per se*, protein content, hectolitre weight and 1,000-kernel weight. In an attempt to identify DNA markers associated with this trait, 100 recombinant inbred lines (RILs) derived from a cross between a hard grain land-race, NP4, and a soft grain variety, HB 208, were screened with 100 ISSR and 360 RAPD primers. Eighteen markers were assigned to seven linkage groups covering 223.6 cM whereas 11 markers remained unlinked. A multiple-marker model explained the percentage of phenotypic variation for kernel hardness as 20.6%, whereas that for protein content, hectolitre weight and 1,000-kernel weight was 18.8%, 13.5% and 12.1%, respectively. Our results indicate that phenotypic expression of kernel hardness is controlled by many QTLs and is interdependent on various related traits.

Keywords Wheat · Kernel hardness (KH) · Quantitative trait loci (QTL) · RAPD · ISSR · Recombinant Inbred lines (RILs)

Introduction

Kernel hardness (KH) is one of the most important quality parameters of wheat grain, having a profound effect on the milling, baking as well as end-use qualities of bread wheat. It refers to the texture of the kernel, in terms of a physically hard or soft endosperm (Giroux and Morris 1998). Hard wheat flour is generally used for bread, whereas soft wheat flour is used for pastries, cakes and confectionaries (Tippless et al. 1994). An assessment of endosperm texture is, therefore, necessary in characterizing the end-use quality of wheat (Bettge et al. 1995). Simmonds et al. (1973) have suggested that hardness is due to a cementing agent between starch and proteins, and is involved with the continuity of the protein matrix and the strength with which it physically entraps starch granules (Stenvert and Kingswood 1977). Other factors which influence the hardness and the milling and baking qualities of wheat grain are the protein content (PC), with a direct effect (Bushuk 1998), and hectolitre weight (HW) and 1,000-kernel weight (TKW), with an indirect contribution (Pomeranz and Williams 1990).

During an attempt to study the genetic control of KH, Symes (1969) has demonstrated that the difference in hardness between a hard and a soft wheat is due to one major gene, designated as *Hardness* (*Ha*), located on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). Baker (1977) and Pomeranz and Williams (1990) have subsequently reported the presence of two major and three minor genes controlling the expression of kernel hardness. The extensive work of several other researchers (Greenwell and Schofield 1989; Jolly et al. 1993) has identified a 15-kDa protein, friabilin, and its correlation with kernel hardness. Friabilin is present on the surface of water-washed starch, is abundant on soft wheat and scarce on hard wheat starch, and is primarily composed of two polypeptides, puroindoline-a and puroindoline-b (Giroux and Morris 1997). However, friabilin does not appear to be specific for soft wheat suggesting that it is also present in the endosperm of hard wheat (Jolly et al. 1993). Friabilin alone thus may not be useful as a marker for grain hardness or softness (Wrigley 1994).

Kernel hardness (KH) is currently measured by methods such as the particle-size index of flour, the time and resistance required to grind grain and the near-infrared

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reflectance spectroscopy (NIR) of whole grain meal (Pomeranz and Williams 1990). Although these methods are useful in determining the texture of bulked grain lots (Morris 1992), they have limitations in determining the composition of mixtures of hard and soft wheat and in assessing the genotypic hardness. Further, phenotypic hardness varies considerably due to the environment affecting the reliable determination of genotypic hardness (Morris 1992). The identification of molecular markers for the reliable assessment of genotypic hardness has, therefore, received considerable attention in recent years.

Using RFLP markers, Sourdille et al. (1996) have reported the presence of a major locus *Xmta9* linked closely to the gene *ha* (hard) on 5DS and seven other minor loci distributed on different chromosomes. Genes for puroindoline a (*pin-a*), puroindoline b (*pin-b*) and grain softness protein (*Gsp-1*), closely linked to the *Ha* (soft) locus, have been shown to be associated with the expression of grain softness (Dubcovsky et al. 1999; Turner et al. 1999). Giroux and Morris (1998) have shown the presence of a single mutation in either protein, a null in *pin-a* or a glycine to serine sequence change in *pin-b*, to be associated with hard grain texture. The single nucleotide change in *pin-b* has been further exploited to create discriminating PCR primers for *pin-a* and *pin-b* sequences (Giroux and Morris 1997). Recently, Dubcovsky et al. (1999) have developed a more-reliable, codominant CAPS (cleavage amplified polymorphic sequence) marker to detect the point mutation in *pin-b* which can be used to determine kernel texture. However, untill now, the emphasis of all the molecular studies has been restricted to the *Ha* locus and there is a need to further understand other genetic factors contributing to the variation for KH beyond that explained by *Ha* (Morris 1998).

PCR-based markers, and inter simple sequence repeat markers (ISSRs) in particular, are extremely useful for tagging agronomically important traits and can further be used for marker-assisted selection (Ammiraju et al. 2001). In this paper, we report the identification of ISSR and randomly amplified polymorphic DNA (RAPD) markers associated with four different traits contributing to KH, namely NIR, PC, HW and TKW.

Material and methods

Plant material

The mapping population consisted of 100 recombinant inbred lines (RILs, at the F_7 generation) derived from a cross between NP4, a hard wheat land race, and HB 208, a soft wheat. These parents also differed with respect to PC, HW and TKW. The RILs were developed following a single-seed descent method at the Directorate of Wheat Research (DWR), Karnal, India. Seeds of nullitetrasomic (NT) and ditelosomic (DT) lines were procured from Dr. B.S. Gill, Kansas State University, USA.

Trait measurements

KH was determined by NIR spectroscopy with a Technicon 400 Infra-analyser (Technicon Corporation, N.Y.) according to the AACC method (AACC 1989). PC was determined by the Kjeldahl method (AACC 1983) while HW was measured by the hectolitre weight-measuring funnel of the SINARFP Auto 6080 moisture analyser by Tecator. TKW was determined by obtaining the weight of 1,000 grains in grams using an electronic counter (Misra and Gupta 1995).

DNA extractions

Total genomic DNA was extracted from 15-day old seedlings of parents and RILs, as well as of NT and DT lines grown in the glass house, by the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1988).

PCR amplification using ISSR and RAPD primers

ISSR reactions were performed according to Ammiraju et al. (2001) using primers from the Biotechnology Laboratory, University of British Columbia, Canada (UBC). RAPD reactions were carried out using 20 ng of genomic DNA, 5 pmol of primer (Operon Technologies, USA) in 1× amplification buffer and 1 unit of *Taq* DNA polymerase (Perkin Elmer) per 25-µl reaction. The PCR protocol was as described by Naik et al. (1998) with slight modification.

All PCR reactions were performed in a PTC-200 thermocycler (MJ Research, USA); the PCR products were electrophoresed on 2% agarose gels and visualized under UV light after staining with ethidium bromide.

Linkage analysis and mapping

From the amplification profiles of RILs using polymorphic primers, data were recorded as the presence or absence of bands since both RAPDs and ISSRs are dominant markers. Linkage relationships among the markers were determined using MAPMAKER version 3.0 (Lander et al. 1997) and linkage groups were constructed at LOD 3.0. The Haldane function was used to convert the recombination frequencies to centiMorgans (Haldane 1919).

Single-marker analysis using a simple linear-regression approach was performed using the QGENE programme to identify marker-trait associations (Nelson 1997). Multiple linear-regression analysis was carried out using QGENE software to determine the cumulative effect of marker loci on the phenotypic variation.

Chromosomal assignment of the markers linked to KH parameters

The assignment of significant markers to specific chromosomes was done through PCR amplification using template DNA from each of the 21 NT lines derived from Chinese Spring. Identification of the arm location was done in the same way using chromosome-specific DT lines of Chinese Spring (Sears 1966; Sears and Sears 1978).

Results

Distribution of kernel hardness in the RIL population

The frequency distribution of NIR value, PC, HW and TKW, in the form of histograms for the RIL population generated from a cross NP4×HB208, is depicted in Fig 1. In the case of NIR value and PC, the progeny showed transgressive segregants in both directions, whereas for HW and TKW it was more towards softness, suggesting that both the parents possess positive and complementary alleles for these traits (Sourdille et al. 1996).

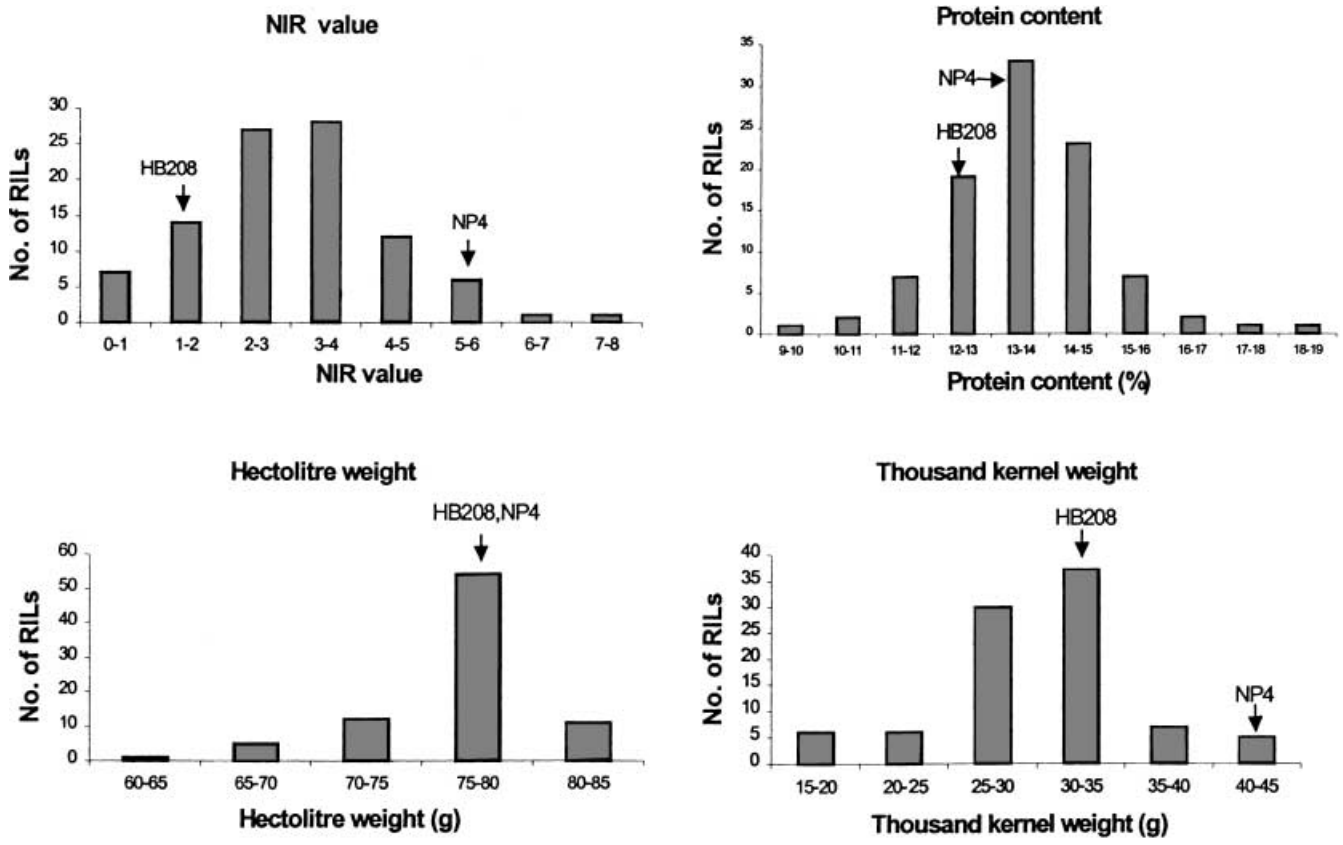

Fig. 1 Frequency distribution of NIR value, PC, HW and TKW in the RIL population of the cross NP4×HB208

Association of DNA markers with the traits

All the ISSR and RAPD primers were initially used for the parental survey, followed by selective genotyping (Lander and Botstein 1989), and putative markers were then attempted with the RIL population.

Twelve ISSR and seven RAPD markers revealing 29 loci segregating in a phenotype-specific manner were further tested for goodness of fit to a 1:1 ratio using chi-square analysis (Table 1A and 1B). Twenty markers segregated in accordance with the expected ratio of 1:1, with probabilities ranging from 0.001 to 0.70. Four markers, $UBC818_{1078,}$ $UBC827_{1350,}$ $UBC836_{872}$ and $OPC11_{650}$, deviated from the expected ratio showing skewed segregation towards one of the parents.

The genetic linkage map was generated with segregating markers using MAPMAKER version 3.0 at LOD 3.0. Eighteen markers were assigned to seven linkage groups, which equals the haploid number of chromosomes in the wheat genome, while 11 markers remained unlinked. All the linked markers covered a 223.6 cM distance, which is quite small as compared to the total map distance of the wheat genome. Linkage group 6 consisted of four markers ($UBC815_{1000,}$ $UBC818_{1078,}$ $UBC827_{1350}$ and $OPJ01_{700}$) covering a maximum map distance of 62.5 cM and was shared by two traits KH and PC, whereas marker $UBC880_{1200}$, contributing to three traits PC, HW and TKW, mapped to linkage group 4. Linkage group 2 consisting of $UBC812_{1000}$ and $OPB15_{880}$ was shared by KH and PC, while linkage group 1 ($UBC880_{1000}$ and $OPA04_{750}$) was also shared by KH and HW. The contribution of linked and unlinked markers to the phenotype indicated the distribution of KH controlling QTLs over different chromosomal regions.

The results of single-marker analysis and multiple-marker analysis for these traits (Table 2) are given below.

Kernel hardness by NIR

Three ISSR markers, $UBC807_{650}$, $UBC873_{880}$ and $UBC880_{1000}$, and four RAPD markers, viz. $OPB15_{880}$, $OPJ\ 01_{700}$, $OPC16_{860}$ and $OPA04_{750}$, were found to be associated with NIR values, with a multiple linear regression of 20.6%. The R^2 values of these markers by single-marker analysis ranged from 8.61% ($P<0.007$) to 3.26% ($P<0.080$). Markers like $OPB15_{880}$, $OPJ01_{700}$ and $UBC807_{650}$ were also associated with PC, $UBC873_{880}$ and $UBC880_{1000}$ with HW, whereas UBC 873_{880} was associated with TKW. $OPA04_{750}$ and $OPC16_{860}$ were unique to KH.

Protein content

A total of 11 markers (eight ISSRs and three RAPDs) showed an association with PC, with a total phenotypic

Table 1

Primer	Sequence	Fragment size (bp)	a:b Segregation	Chi-square	Probability
A: segregation data of polymorphic ISSR primers					
UBC807	AGA GAG AGA GAG AGA GT	650	36:55	4.42	0.02–0.05
UBC811	AGA GAG AGA GAG AGA GGA	870	42:58	2.56	0.10–0.20
UBC835	AGA GAG AGA GAG AGA GYT	700	35:48	4.58	0.02–0.05
UBC836	AGA GAG AGA GAG AGA GYA	872	67:30	13.78	–
UBC812	GAG AGA GAG AGA GAG AA	1,000	36:56	4.64	0.02–0.05
UBC812	GAG AGA GAG AGA GAG AA	800	35:57	5.48	0.02
UBC827	ACA CAC ACA CAC ACA CG	1,350	17:75	34.28	–
UBC827	ACA CAC ACA CAC ACA CG	1,300	60:32	8.48	0.001–0.01
UBC848	CAC ACA CAC ACA CAC ARG	890	48:52	0.16	0.50–0.70
UBC848	CAC ACA CAC ACA CAC ARG	400	44:56	1.44	0.20–0.30
UBC856	ACA CAC ACA CAC ACA CYA	700	39:60	4.42	0.02–0.05
UBC856	ACA CAC ACA CAC ACA CYA	610	42:57	2.26	0.10–0.20
UBC856	ACA CAC ACA CAC ACA CYA	590	53:46	0.5	0.30–0.50
UBC818	CAC ACA CAC ACA CAC AG	1,078	64:29	12.74	–
UBC815	CTC TCT CTC TCT CTC TG	1,000	61:34	7.54	0.001–0.01
UBC815	CTC TCT CTC TCT CTC TG	900	56:39	3.14	0.05–0.10
UBC815	CTC TCT CTC TCT CTC TG	800	42:53	1.46	0.20–0.30
UBC815	CTC TCT CTC TCT CTC TG	700	41:54	1.94	0.10–0.20
UBC873	GAC AGA CAG ACA GAC A	880	54:44	1.04	0.30
UBC880	GGA GAG GAG AGG AGA	1,200	45:53	0.68	0.30–0.50
UBC880	GGA GAG GAG AGG AGA	1,000	42:56	2.0	0.10–0.20
B: segregation data of polymorphic RAPD primers					
OPA 04	AATCGGGCTG	750	63:34	8.5	0.001–0.01
OPB 15	GGAGGGTGTT	880	38:48	2.96	0.05–0.10
OPC 11	AAAGCTGCGG	1,000	39:59	4.04	0.02–0.05
OPC 11	AAAGCTGCGG	650	74:24	25.04	–
OPC 16	CACACTCCAG	860	48:42	1.36	0.10–0.20
OPH 12	ACGCGCATGT	900	44:52	0.8	0.30–0.50
OPI 20	AAAGTGCGGG	690	37:56	4.1	0.02–0.05
OPJ 01	CCCGGCATAA	700	53:32	7.4	0.001–0.01

Y=C,T; R=A,G

Table 2 Percentage of phenotypic variation in the marker-trait associations

Trait	Markers	R^2 with single marker effect ×100	Probability	R^2 with multiple-marker model ×100
NIR value	$OPB15_{880}$	8.61	0.007	20.6
	$OPJ01_{700}$	5.97	0.026	
	$OPC16_{860}$	5.36	0.031	
	$UBC807_{650}$	4.79	0.040	
	$UBC873_{880}$	4.29	0.045	
	$UBC880_{1000}$	3.62	0.066	
	$OPA04_{750}$	3.26	0.083	
Protein content	$OPB15_{880}$	10.46	0.003	18.8
	$UBC815_{1000}$	8.87	0.003	
	$OPI\ 20_{690}$	8.48	0.005	
	$UBC856_{610}$	6.4	0.013	
	$UBC827_{1300}$	5.85	0.022	
	$UBC807_{650}$	5.69	0.025	
	$OPJ01_{700}$	5.07	0.042	
	$UBC818_{1078}$	4.46	0.047	
	$UBC812_{800}$	4.39	0.050	
	$UBC812_{1000}$	4.28	0.053	
	$UBC880_{1200}$	3.09	0.090	
Hectolitre weight	$UBC880_{1000}$	7.1	0.016	13.5
	$UBC873_{880}$	6.41	0.023	
	$UBC880_{1200}$	3.9	0.077	
1,000-kernel weight	$UBC873_{880}$	6.41	0.016	
	$UBC880_{1200}$	5.83	0.023	
	$UBC856_{590}$	4.97	0.035	12.1

contribution of 18.8%. R^2 values by single-marker analysis ranged between 10.46% ($P<0.003$) to 3.09% ($P<0.090$). Most of the markers that were associated with PC also exhibited associations with KH; for example, markers like $OPB15_{880}$, $OPJ01_{700}$ and $UBC807_{650}$ were shared by both traits. $UBC880_{1200}$ with a low contribution to PC was shared by both HW and TKW, while $OPI20_{690}$, $UBC815_{1000}$, $UBC856_{610}$, $UBC827_{1300}$, $UBC818_{1078}$, $UBC812_{800}$ and $UBC812_{1000}$ were unique to PC.

Hectolitre weight and 1,000-kernel weight

Three ISSR markers, $UBC880_{1000}$, $UBC873_{880}$ and $UBC880_{1200}$, were found to be associated with HW, with 13.5% of the total phenotypic variation, while $UBC873_{880}$, $UBC880_{1200}$ and $UBC856_{590}$ with a 12.5% contribution were found to be associated with the total phenotypic variation in TKW. Markers like $UBC873_{880}$ and $UBC880_{1200}$ contributed to both traits. $UBC856_{590}$ was observed to be a unique marker associated with TKW only and not to any other trait in this analysis. The total number of markers that were associated with these two traits, and the total phenotypic variation estimated by the multiple-marker model, were found to be less as compared to that of KH and PC.

Chromosomal localization of markers

The chromosome assignment of markers associated with all the traits was done using the NT and DT lines. This analysis indicated that two markers, $UBC807_{650}$ contributing to KH and PC, and $UBC812_{800}$ contributing to PC alone, were present on the short arm of chromosome 6B and the long arm of 2B, respectively. Other ISSR and RAPD markers could not be assigned to chromosomes, as they did not show any amplification in Chinese Spring, in which background the NT and DT lines were established.

Discussion

Markers associated with KH and related traits

All previous reports and the inheritance studies have emphasized the presence of a major locus, *Ha*, for KH, with some additional minor loci. However, the *Ha* gene alone cannot explain all of the variation for this trait, indicating the presence of other minor loci that are also involved in its phenotypic expression. We have identified 16 PCR-based markers associated with all four traits contributing to kernel hardness for the first time (Table 2). Thus, the presence of a number of markers with minor effects on phenotypic variation indicates the complexity of this trait and strongly supports the involvement of additional chromosomal regions apart from *Ha* on 5DS.

Sourdille et al. (1996) have also reported four additional regions on chromosomes 2A, 2D, 5B and 6D contributing to hardness, while three others having interaction effects are located on chromosomes 5A, 6D and 7A. Joppa et al. (1997) have shown the presence of a major locus on 6BS for high grain-protein content (GPC) while Campbell et al. (1999) have found chromosome 2B to contain QTLs for flour-protein quantity. Ammiraju et al. (2001) have demonstrated the presence of loci for TKW on chromosomes 6B and 2D. Here, for the first time, we report the identification of chromosomes 2B and 6B carrying loci controlling KH and PC. The localization of the remaining markers could not be determined, as explained in the results; however, they may be localized to previously reported chromosomal locations, or even to new locations, in the case of QTLs for KH, PC and other traits.

KH, PC, HW, TKW: trait interrelations

According to Bushuk (1998), KH and PC are probably interdependent, and hard wheat is generally high in protein content whereas soft wheat is low in protein content. Some marker loci involved in kernel hardness are mapped to the same regions as storage proteins (Sourdille et al. 1996); however, no positive correlation exists between KH and PC in the mapping population used by them. It is, therefore, interesting to identify and compare the QTLs for both traits in order to support the influence of PC on KH. We have shown that three markers are associated with both KH and PC, indicating that a few loci contributing to KH and PC are linked to each other in the present population. This is also supported by the identification of chromosomes 2B and 6B carrying marker loci for both KH and PC. Interestingly, in our studies, most of the markers are associated with more than one trait. Moreover, the linkage groups have also been shared by all these traits, indicating the probable sharing of loci on different chromosomes. Further studies on the mapping of these loci and the identification of additional markers contributing to the phenotypic variation would throw more light on the interrelations of these contributing traits. Efforts in this direction are now in progress.

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