Theor Appl Genet (2002) 106:19–27
DOI 10.1007/s00122-002-0971-8

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Mapping QTLs for grain hardness and puroindoline content in wheat (*Triticum aestivum* L.)

Received: 24 September 2001 / Accepted: 2 March 2002 / Published online: 21 June 2002
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Abstract Genes for puroindoline-a (Pin-a), puroindoline-b (Pin-b) and grain-softness proteins (GSP) have been shown to be linked to the dominant *Ha* locus responsible for the soft texture of the grain. Though linkage has been demonstrated of the puroindoline genes to the *Ha* locus, there is no clear evidence that puroindoline content is the product of the gene *Ha*. A segregating population of 115 recombinant inbred lines (RILs) originating from a cross between the hexaploid Synthetic wheat (*Triticum durum* × *Aegilops tauschii*, W 7984) and the cultivar 'Opata' (M 85) was studied in two different experimental years to detect Quantitative Trait Loci (QTLs) for three traits: grain hardness (Hard), puroindoline-a (Pin-a) and puroindoline-b (Pin-b) contents. The detection of QTLs was performed using marker linear regression. Negative correlation coefficients (–0.86 and –0.80) were identified between grain hardness and puroindoline content (a and b, respectively) on data obtained in 1996. Results obtained in 1999 confirmed the negative correlation between Hard and Pin-a (–0.73); however a positive correlation coefficient was found with Pin-b content (0.41). Total phenotypic variation explained by each QTL was calculated (R^2). For each of the Hard, Pin-a and Pin-b traits one major QTL was detected on the short arm of chromosome 5D, located close to the *mta9* allele (puroindoline-a). For the first year (1996) the QTL in this region explained around 63% of the phenotypic variability in grain hardness, 77% in Pin-a and 45% in Pin-b contents. These values were confirmed in trials carried out in 1999 with a R^2 value of 0.71, 0.72 and 0.25 for Hard, Pin-a and Pin-b, respectively. In 1996 and 1999 a second major QTL was detected for grain hardness on the long arm of the same chromosome. Present results indicate that it cannot be definitely concluded that puroindoline content represents a linear explanation for variations in grain hardness.

Keywords Puroindolines · Kernel hardness · QTL · *Triticum aestivum* L.

Communicated by J. Snape

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Introduction

The texture of the endosperm results mostly from the expression of a major gene designated Hardness (*Ha/ha*) located on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). The fact that grain hardness (Hard) is mainly due to a single gene was demonstrated by Symes (1965); however, the same study also indicated the existence of minor genes that modify the action of the main hardness gene. Although *Ha/ha* has been reported to be important for kernel hardness, its gene product is not yet known with certainty. Friabilins or grain-softness proteins (GSP) were revealed to be associated with grain softness and hardness (Greenwell and Schofield 1986; Jolly et al. 1993; Bettge et al. 1996). Greenwell and Schofield (1986) reported that friabilins were abundant in soft, scarce in hard and absent in durum water-washed, wheat starch granules. The GSP or friabilins are now known to contain three main components: puroindoline-a, puroindoline-b and GSP-1 (Turner et al. 1999). Puroindolines are basic cysteine-rich proteins with a molecular mass of about 13–15 kDa that are characterised by a unique tryptophan-rich hydrophobic domain. Two isoforms, namely Puroindoline-a (Pin-a) and Puroindoline-b (Pin-b), have been identified (Blochet et al. 1991, 1993). The lipid-binding properties of puroindolines are of major importance for the process-

ing and end-use of bread wheat (*Triticum aestivum* L.). Moreover, grain hardness seems to be due to the tightly linked genes Pin-a and Pin-b, and both Pin-a and Pin-b are apparently required for the expression of grain softness (Giroux and Morris 1998). However, this relationship has not been verified for some Australian cultivars (Turnbull et al. 2000). Hard endosperm was correlated with either of the two being mutated from their wild allelic state. The first mutation to be described was a glycine to serine change in the puroindoline b polypeptide (Giroux and Morris 1997). Giroux and Morris (1998) demonstrated that the second mutation is a null allele in puroindoline a, with no protein or mRNA present. More recently five additional hardness mutations in puroindoline b have been described (Lillemo and Morris 2000).

Results presented by Sourdille et al. (1996) and Giroux and Morris (1997) have shown that the *Xmta9* locus was associated with grain hardness. *Xmta9* is a cDNA coding for Pin-a (Gautier et al. 1994). Recently, Turner et al. (1999) mapped *XWGS/1-D13* (a 5D specific sequence of GSP-1 genes), *Xmta9* (Pin-a) and *Xmta10* (Pin-b) at the same locus using the ITMI (International Triticeae Mapping Initiative) population. A study using a *Triticum monococcum* mapped population has shown that *Gsp-A^{m}1* and *Pina-A^{m}1* were completely linked to *Pinb-A^{m}1* in the distal region of the short arm of chromosome 5A (Tranquilli et al. 1999). The three genes are physically located in a single BAC fragment. Gautier et al. (2000), using a PCR approach, showed that puroindoline genes were present in diploid and hexaploid *Triticum* species but absent in tetraploid species.

Both Pin-a and Pin-b content has been shown to have a significant influence on dough properties (Dubreil et al. 1998a). Multilocal trials revealed that grain hardness and Pin-b content traits were highly heritable (Igrejas et al. 2001). These studies have demonstrated the significant roles played by puroindolines in breadmaking, i.e. that Pin-a and Pin-b are involved in dough strength (W), bread notation and loaf volume. An attempt to verify the relationship between puroindolines and endosperm texture has recently been reported through genetic transformation in rice, showing that expression of the wild-type alleles of Pin-a and Pin-b resulted in small effects on grain softness (Krishnamurthy and Giroux 2001).

The present study was aimed at finding regions of the genome involved in the control of puroindoline content in a genotyped population segregating for grain hardness, for which a high-density molecular map is available.

Materials and methods

Plant material

One-hundred-and-fifteen recombinant inbred lines (RILs) (Nelson et al. 1995) were used in the present study in order to test the presence of Quantitative Trait Loci (QTLs) over the whole genome. The segregating population was obtained from a cross between a soft line, W7984 ('Synthetic', Synthetic amphihexaploid wheat derived from a cross between *Triticum tauschii* and Altar 84 durum), and Opata 85 ('Opata'), hard red spring wheat RILs (F7/8 generation). Each RIL was grown in two replicates under field conditions at the experimental station of the 'Institut National de la Recherche Agronomique' (INRA) in Clermont-Ferrand, France, in 1996 and 1999. For 2 years analysed seeds were harvested separately for each replicate and stored in a cold room.

Determination of protein content and kernel hardness

Grain was milled using a Cyclotec lab mill (Tecator) for wholemeal production. The flour protein content and kernel hardness of the 115 RILs were estimated by Near Infrared Reflectance (NIR-Percon Inframatic 8620) according to AACC Approved Methods 39-70A (American Association of Cereal Chemists 1995).

Extraction and determination of puroindoline contents

The puroindolines of the wheat flour (0.250 g) were extracted for each RIL replicate as described previously (Igrejas et al. 2001). Puroindolines were extracted from wheat flour using Tris buffer (0.1 M Tris-HCl pH 7.8, 5 mM EDTA, 0.25 M NaCl) containing 2% Triton X-114. Immunochemical ELISA analysis was used to estimate puroindoline content as previously described (Turnbull et al. 2000; Igrejas et al. 2001).

Statistical and QTL analyses

All statistical and QTL analyses were performed on data from each experimental year. The genetic map derived from the 'Synthetic' × 'Opata' cross (ITMImap) covered 3,488 cM with 265 markers, specially choose among the 1,125 available markers, to well-cover the 21 chromosomes. More than 70% of the choosen markers had data points for the 115 RILs. The reference ITMImap as reported by Leroy et al. (1997), was obtained using MapMaker/Exp. version 3.0b (Lander et al. 1987). CentiMorgan (cM) values were calculated using the Haldane mapping function (Haldane 1919). Additional segregating data, especially those concerning microsatellite markers (SSRs), were kindly provided by M. Röder, IPK-Gatersleben (Röder et al. 1998). Associations between markers and traits of kernel hardness, Pin-a and Pin-b content, were investigated using one-way ANOVA. An error risk of $P = 0.001$ was used for testing individual markers in order to achieve a global risk of about 0.05. The error risk P value was obtained through preliminary tests using several P values of individual threshold, the actual map, and a simulated random vector of quantitative traits. This resulted in the conclusion that $P = 0.001$ leads to a genome wise type-I error of 5% out of 1,000 replicates. A set of unlinked significant markers was then used as covariates when scanning each linkage group for the presence of QTLs using marker regression with either a one-QTL (Kearsey and Hyne 1994) or two-QTL model (Hyne and Kearsey 1995), the most significant markers from the other groups being used as covariates as proposed by Jansen and Stam (1994). Confidence intervals (CI) of QTLs were estimated using 1,000 (one-QTL model) or 100 bootstrap resamplings for the two-QTL models (Visscher et al. 1996).

Total phenotypic variation explained by each QTL was estimated as a^2/Vp, a being the additive value of the QTL and Vp the phenotypic variation, as suggested by Kearsey and Hyne (1994).

Results

Distributions of the grain parameters

A preliminary statistical analysis of the recombinant lines was applied to compute the mean and range of the values for all variables reported in this study (Table 1).

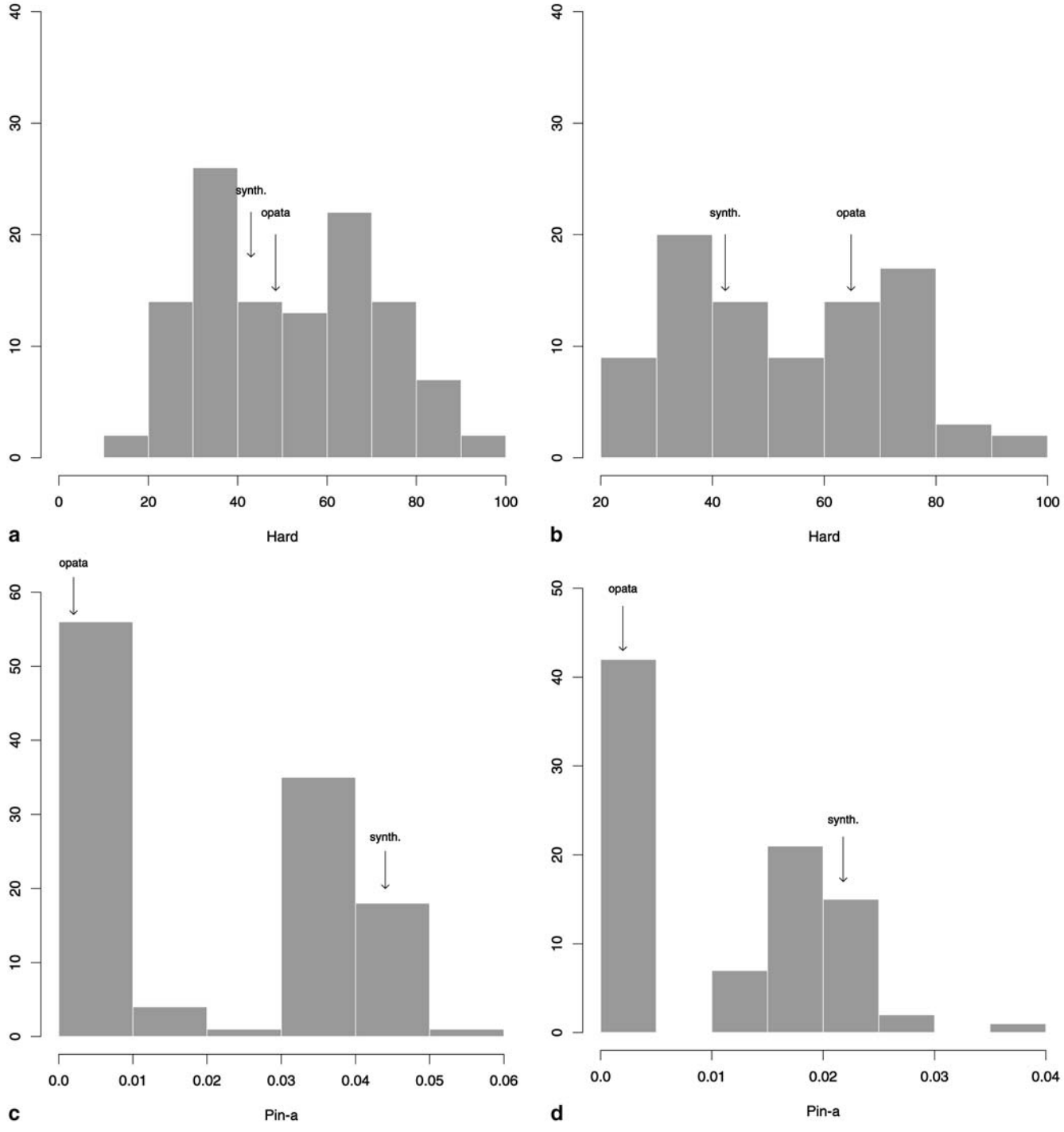

Fig. 1 Distribution (expressed in frequency) of the ITMImap genotyped lines for kernel hardness (*a*, *b*), Pin-a (*c*, *d*) and Pin-b (*e*, *f*) among the population of recombinant inbred lines in the 1996 and 1999 years, respectively. The measurements of the parents (indicated with an *arrow*) 'Synthetic' and 'Opata' were denoted by *synth* and *opata*, respectively

The distribution of the RILs as well as parental scores for grain hardness, Pin-a and Pin-b, are presented in Fig. 1. While the two parents of the ITMI population do not differ widely for hardness ('Synthetic': 43, 42, 'Opata': 49, 65 in 1996 and 1999, respectively) the progeny had kernel-hardness scores ranging from 16 (soft) to 100 (very hard) in 1996, and from 22 to 100 in 1999 (Fig. 1a and b). There were two subgroups in the kernel hardness distribution: the first group being composed of soft to medium-hard kernel phenotype RILs, including the 'Synthetic' variety, and the second sub-group ranged from medium-hard to very hard RILs and including 'Opata' (Fig. 1a and b). With respect to kernel hardness (Hard), both parental lines showed similar values for 'Synthetic' and 'Opata'; however, for protein content

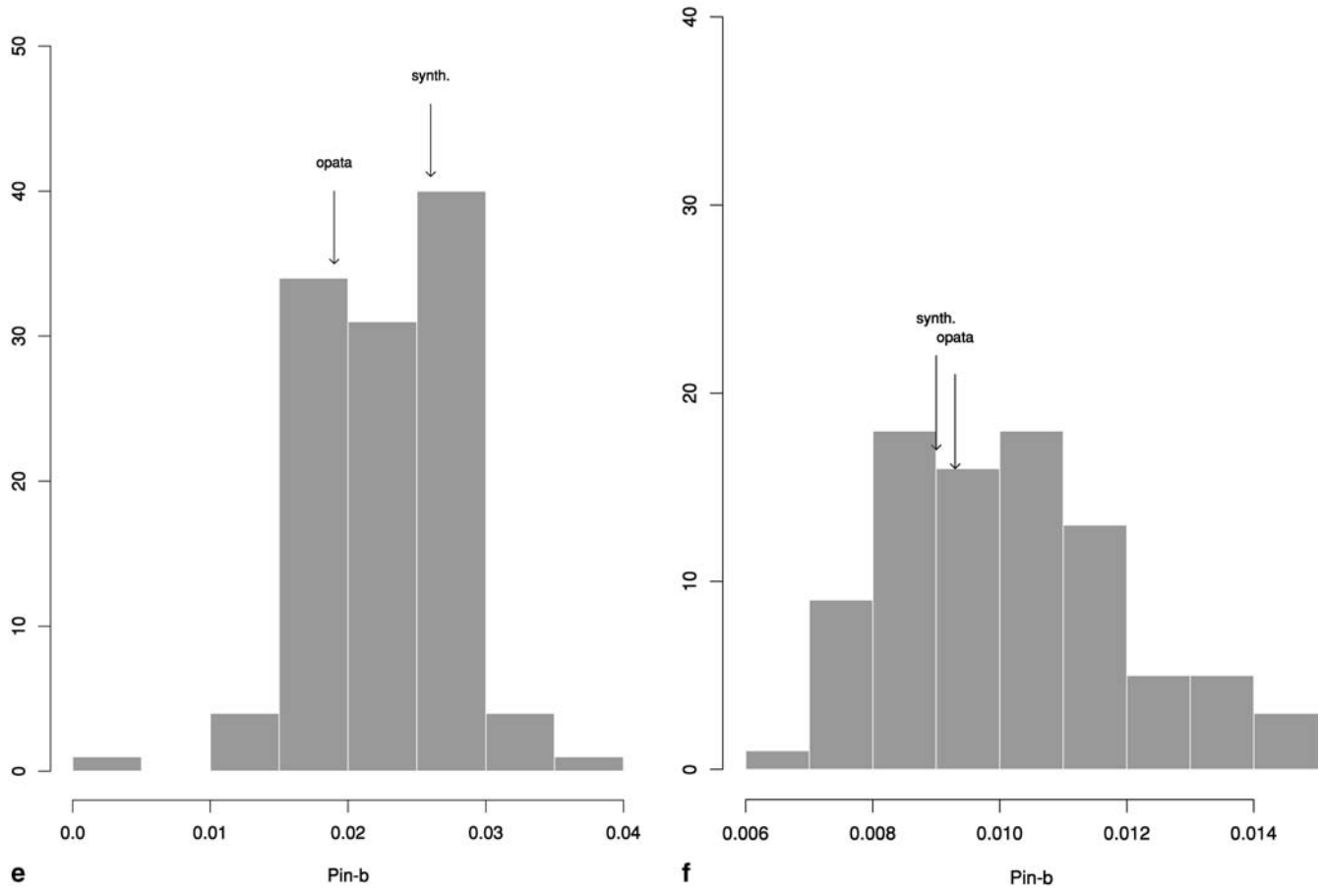

Fig. 1 (continued)

Table 1 Main statistical values (average and range of variables) of the puroindoline contents, protein content and grain hardness of the ITMI population derived from 'Synthetic' and 'Opata' progenitors

Variables (abbreviation)		Average		Minimum		Maximum		'Synthetic'		'Opata'	
Puroindoline-a content[a]	Pin-a	0.020[b]	0.011[c]	0.002[b]	0.002[c]	0.056[b]	0.039[c]	0.044[b]	0.022[c]	0.002[b]	0.002[c]
Puroindoline-b content[a]	Pin-b	0.023	0.010	0.002	0.007	0.036	0.015	0.026	0.009	0.019	0.009
Protein content (% dw[d])	Prot	15.4	14.8	12.4	12.2	20.5	18.4	17.8	16.5	12.6	12.4
Hardness (%)	Hard	52	53	16	22	100	100	43	42	49	65

[a] Units of puroindolines content: mg g^{-1} of dry flour
[b] Data obtained in 1996
[c] Data obtained in 1999
[d] Dry weight

(Prot) the two varieties showed highly contrasted values ('Synthetic', 17.8 and 16.5, and 'Opata 12.6 and 12.4%, respectively, in 1996 and 1999) (Table 1). This bimodal shape of kernel-hardness distribution of the lines derived from the ITMI population emphasized the presence of a major gene controlling hardness already identified by Symes (1965). A similar distribution shape was observed for Pin-a (Fig. 1c and d). Pin-a values were very different for the two parents; 'Synthetic' had a value of 0.044 while Pin-a could not be detected in 'Opata' (below the detection limit). Therefore 'Opata' can be considered a null Pin-a allelic variant. The Pin-b content also had a non-Gaussian shape (Fig. 1e and f) with a bimodal distribution, whereas protein content had a Gaussian distribution (data not shown). The 'Synthetic' variety showed a higher level than 'Opata' in Pin-a and Pin-b content in 1996 (Fig. 1c to e). In 1999 the values obtained for Pin-b were very similar for these two varieties ('Synthetic' = 0.0090 and 'Opata' = 0.0093) (Fig. 1f).

Correlations between grain parameters

The first-year (1996) grain hardness was negatively correlated with Pin-a and Pin-b content (R^2 = –0.86 and –0.80, respectively). Pin-a content was positively corre-

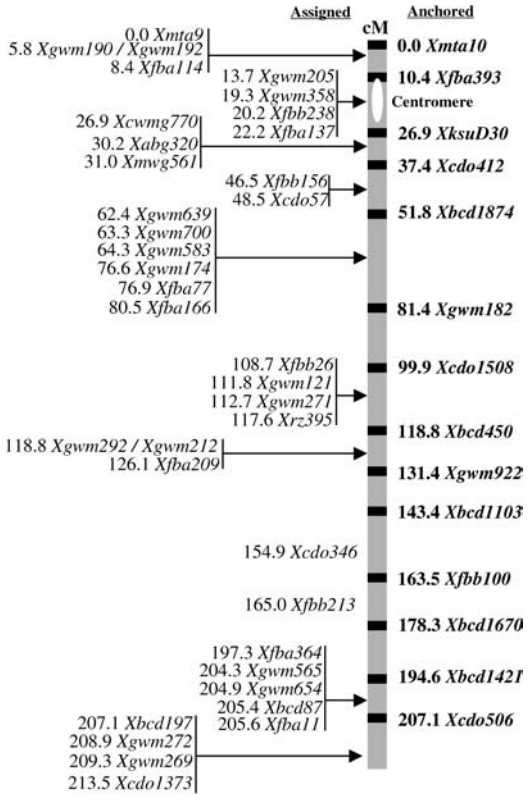

Fig. 2 RFLP molecular linkage of wheat chromosome 5D. The short arm of the chromosome is at the top. At the right are the anchor markers used to build the chromosome framework. The order of markers used as anchors has been checked using the MAPMAKER 'ripple 2 5"' command. At the left are assigned markers in the most probable interval at LOD ≥ 3.0 and <35% percentage recombination. All the marker genetic links have been checked using the MAPMAKER "links any 3.0 35" command. Genetic distances are in centiMorgans (cM) using the Haldane function (Haldane 1919)

lated with Pin-b content (0.77). These traits exhibited correlation coefficients higher than those previously reported by Dubreil et al. (1998a) and Igrejas et al. (2001). Protein content was positively correlated with grain hardness (0.35 in 1996 and 0.32 in 1999), and negatively, but not significantly, with puroindoline content (data not shown). In 1999 grain hardness was also negatively correlated with Pin-a (–0.73); however, a correlation value of 0.41 was found for Pin-b. No significant correlation was observed between protein content and puroindoline content. For all traits, correlation coefficients between the years were high: 0.76 for protein content, 0.82 for grain hardness and 0.83 for puroindoline a. Only puroindoline b was negatively, but not significantly, correlated (–0.25).

QTL analysis

Figure 2 shows the molecular markers involved in our study of chromosome 5D and their map locations. Several markers were associated with the parameters analysed in the 1996 and the 1999 trials. Two markers were found to be significantly associated with Hard: *Xfba393* (R^2 = 33.3; 24.9) and *Xmta9* (R^2 = 62.6; 60.2), respectively, in the 1996 and the 1999 seasons. The same markers were also found significantly associated with Pin-a content: *Xfba393* (R^2 = 36.4; 30.2) and *Xmta9* (R^2 = 81.0; 63.9). The only marker significantly associated with Pin-b content was *Xmta9* (R^2 = 49.5; 31.0).

The map of the QTLs having the main effects in 1996 and 1999 is given for Hard (Fig. 3a and b), Pin-a (Fig. 3c and d) and Pin-b (Fig. 3e and f) content. Table 2 presents the average bootstrap values and the 95% confidence interval (CI) for locations. Two significant QTLs were detected for grain hardness: one at position 1.1, i.e. close to *Xmta10*, and the second at position 118.3, linked to the *Xbcd450* locus (proximal part of chromosome 5DL). The most important QTL for the Hard trait (close to the locus *Xmta10*) had an additive value AV = –15.5 and –16.2 with an R^2 = 0.63 and 0.71 in 1996 and 1999, respectively. The second major QTL close to the locus *Xbcd450* had AV = 6.3 and 5.9 with an R^2 = 0.10. A similar QTL location was found for Pin-a; the most important QTL (position 0.8 and 2.0) had an R^2 = 0.77 and 0.72. We also identified one QTL on this chromosome for Pin-b content, but in the 2nd year its location was different from Pin-a and Hard. *Xmta9* and *Xmta10* were very tightly linked markers and could not be differentiated, giving

Table 2 Average bootstrap values (BV) and 95% confidence intervals (CI) of the two-QTL model. QTL location is expressed in cM from the origin, as additive values, CI is in brackets. Total phenotypic variation explained by R^2 = coefficient of determination, AV = additive value of the QTL. Other abbreviations as presented in Table 1

Year	Parameter	QTL1 Location (cM) BV [CI]	AV	R^2	Allele[a]	QTL2 Location (cM) BV [CI]	AV	R^2	Allele[a]
1996	Hard	1.1 [0; 4.7]	15.5	0.63	o	118.3 [33.0; 227.1]	6.3	0.10	s
	Pin-a	0.8 [0; 3.7]	0.016	0.77	s				
	Pin-b	0.7 [0; 4.5]	0.004	0.45	s				
1999	Hard	1.3 [0; 7.3]	16.2	0.71	o	131.7 [106.1; 330.1]	5.9	0.10	s
	Pin-a	2.0 [0; 6.4]	0.008	0.72	s				
	Pin-b	5.2 [0; 143.1]	0.001	0.25	s				

[a] Positive allele effect of 'Synthetic' "s" or 'Opata' "o"

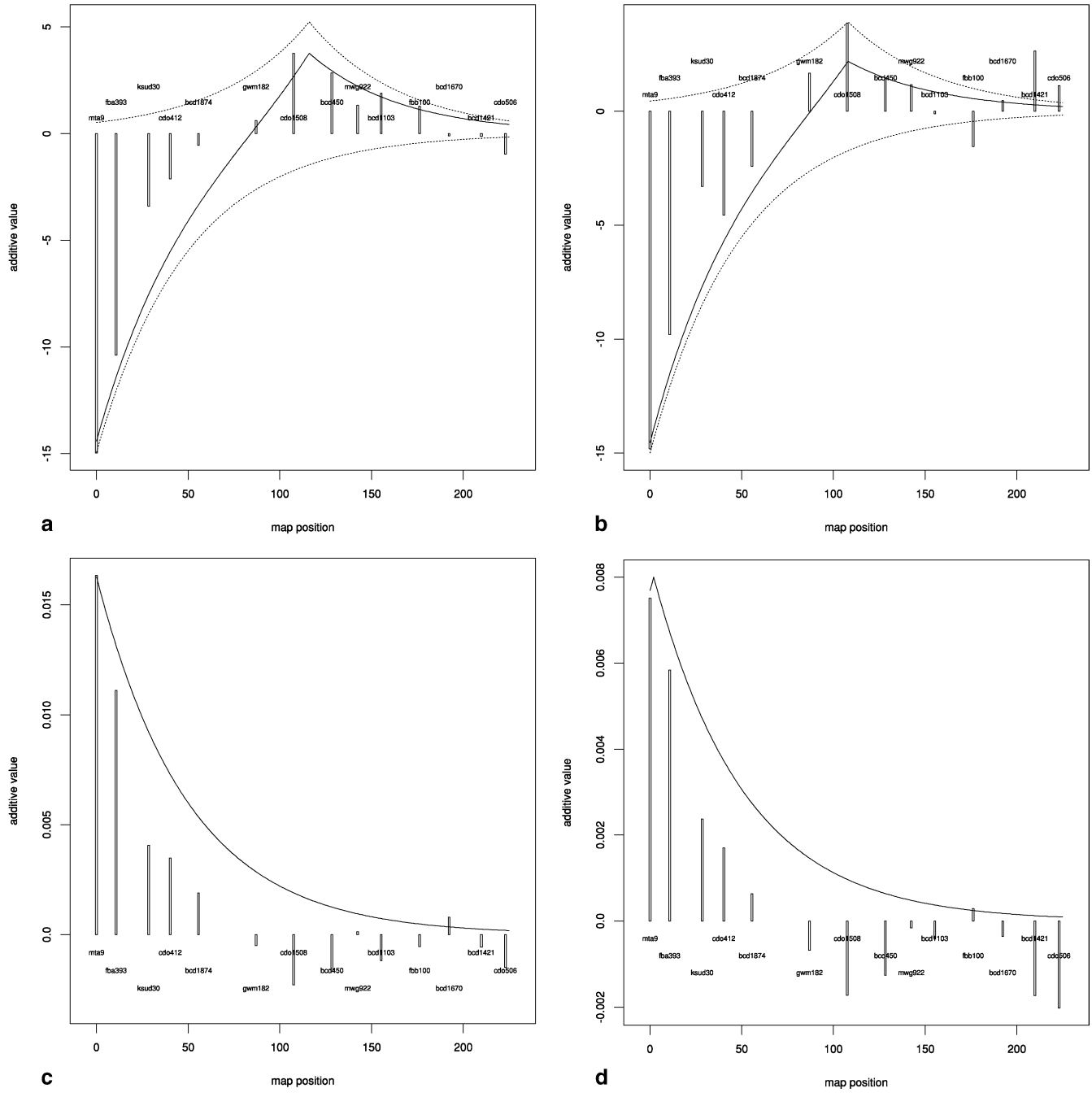

Fig. 3a–f Adjustment of the two-QTL model by 'marker regression' on chromosome 5D for the ITMImap cross. **a** and **b** Hard; **c** and **d** Pin-a; and **e** and **f** Pin-b. *Dotted lines* are the regression lines for each QTL, the *full line* is the sum of the two additive effects. Positions of the anchor markers are given along the abscissa in cM

the same major QTL for all three parameters, Hard, Pin-a and Pin-b content.

Discussion

Correlation coefficients between parameters for the 2 years analysed were significant except for Pin-b content. The significant correlations between Pin-a and Hard in 1996 and 1999 could result in the high genetic influence on these two parameters. No clear explanation was found for the strong Pin-b variation observed between the 2 years. The lack of reproducibility between 1996 and 1999 could also explain the absence of a relationship between Pin-a and Pin-b contents. Although Pin-b content had low variation in 1999 the genetic influences were not absent since the same chromosome (5D) was involved in the explanation of this trait. As significant markers were only detected on chromosome 5D, it was not necessary to use covariates in marker regression. Rather one-QTL and two-QTL models were tested on chromosome 5D. The two-QTL model was found to be

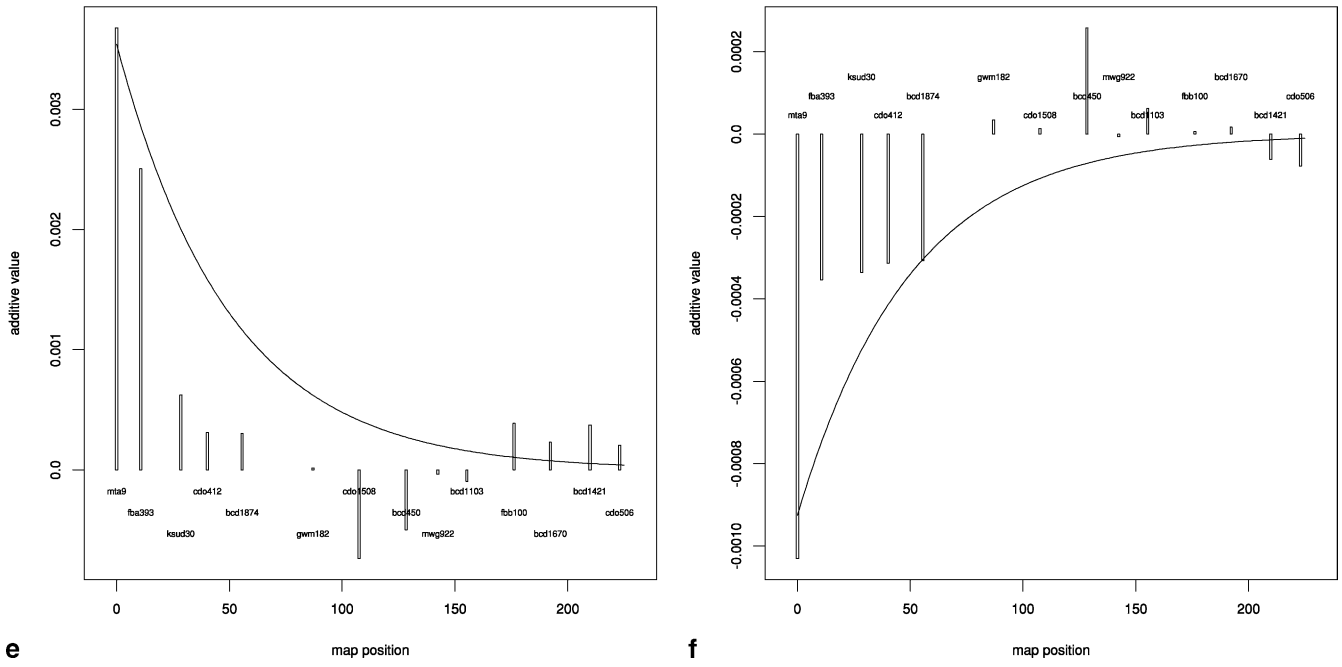

Fig. 3 (continued)

highly significant when tested against the one-QTL model for the Hard trait. The large confidence intervals of the second significant QTL (QTL2), results in the fact that its effect is rather low ($R^2 = 0.10$) as compared to the first one. The positive effect of QTL2 was associated to Synthetic, whereas it was associated to Opata for QTL1. A molecular marker (*Xmta9*) located on this region of chromosome 5DS has been previously reported to be associated with kernel hardness (Sourdille et al. 1996; Perretant et al. 2000). Our results confirm the relationship to the *Xmta9* locus (Pin-a) and the *Xmta10* locus (Pin-b), but revealed a second significant QTL, located on the same chromosome (Fig. 2). In fact, in 1996, the most-important chromosome region with QTLs for all traits was linked to the *Xmta9* locus, and explains around 63, 77 and 45% of the phenotypic variation in Hard, Pin-a and Pin-b content, respectively (Table 2). For the 1999 trial, the same *Xmta9* locus explains 71, 72 and 25% of the variation in Hard, Pin-a and Pin-b content, respectively. This locus (*Xmta9*) has been reported to account for around 63% of the phenotypic variability in grain hardness (Sourdille et al. 1996). Locus *Xmta10* explains 59% of the total phenotypic variation in grain hardness, 79% of Pin-a and 49% of Pin-b content. The tight linkage of these two markers, respectively *Xmta9* and *Xmta10*, makes it difficult to determine which was associated with the major QTL. Increasing the size of the population would allow an increase in the fine mapping of QTL locations.

Nevertheless, when QTL analyses were carried out on the whole genome, the main QTL was only observed on the short arm of chromosome 5D for all parameters analysed (grain hardness, Pin-a and Pin-b content). The two minor QTLs reported by Sourdille et al. (1996) on chromosomes 2A and 2D were not detected on this data set, obtained from a different year of harvest. No major QTL was found for protein content. The major result of our study is the identification of the similar QTL location for kernel hardness and Pin-a content; each trait having been assessed using a different phenotyping method (NIR and ELISA assay) (Fig. 3). Although not significant ($0.05 < P < 0.10$), a minor QTL was also observed on the long arm of chromosome 5D for puroindoline a (Fig. 3c and d), in the same location where it revealed the second QTL (QTL2) for grain hardness. The second QTL found for grain hardness on the 5DL chromosome may result from associations of free polar lipids controlled by genes (*Fpl-1* and *Fpl-2*) that were also associated with grain hardness (Morrison et al. 1989). The lipid-binding properties of puroindolines, which have been reported to be strongly associated with membrane lipids (Dubreil et al. 1998b), could explain the minor QTL detected for Pin-a content. This minor QTL was found on the same chromosome arm as that reported for the free polar lipids gene (*Fpl-1*).

Since Pin-a and kernel hardness have an identical QTL, Pin-a content might explain variations in grain hardness. In our progeny we confirmed one result reported by Giroux and Morris (1997) that, in the absence of Pin-a, the wheat samples had a kernel hardness ranking from hard to very hard. This suggests a very strong relationship between the two traits, which could explain the relationship between Pin-a and *Ha*. However, Igrejas et al. (2001), evaluating a multilocal collection of European cultivars and advanced lines, found Pin-b content to be more closely correlated with grain hardness than Pin-a content. Moreover, the same study also revealed the presence of a null allele for Pin-a in the collection, and a high Pin-a content was found in both hard and soft lines. In this case the hard wheats could be the result of serine mutation. This single mutation probably has no major consequences for antibody/antigen recognition, which

meant that our ELISA would be unable to detect the Pin-b mutation. Because hardness could result from a loss of function due to serine mutation, when hard wheats are serine-type Pin-b mutants, no association can be revealed with Pin-a and Pin-b content and hardness phenotypes. The present study confirms that the hard allele *ha* is completely linked with the null Pin-a allele described by Giroux and Morris (1997). This null Pin-a mutant, which is observed in many hard varieties, was associated with the Pin-b allele without the glycine to serine mutation (Giroux and Morris 1997, 1998).

Conclusion

The presence of two major QTLs for kernel hardness indicates that this trait is under the control of independent sets of genes on the same chromosome. Both Pin-a and Pin-b content is controlled by the same major QTL that could also result from the complex of components that make up these two protein fractions. These preliminary results are the first genetic approach to puroindoline content, although the QTLs need to be confirmed using other mapping populations. Recent two-dimensional electrophoresis of puroindolines has proved that both Pin-a and Pin-b fractions are composed of several spots, some of which remain to be identified (Branlard et al. 2002). However, puroindolines may have a functional effect on softness if they differ in their amino-acid sequence as reported by Giroux and Morris (1998). The results of this survey show that it is not possible to conclude that puroindoline content (Pin-a and Pin-b) alone explains grain hardness. Other molecular parameters and other interactions are probably responsible for, or involved in, variations in grain hardness. Direct sequencing of cDNA and protein fractions are presently being carried out to test this hypothesis.

Acknowledgements The authors thank M. Röder for providing sequencing data of the gwm SSRs markers mapped on the ITMI population. We also like to thank B. Charef for his contribution to setting out molecular mapping, and Mireille Dardevet and René Saccomano for their technical help with cultivation.

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