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QTL analysis for grain protein content using SSR markers and validation studies using NILs in bread wheat

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Abstract QTL interval mapping for grain protein content (GPC) in bread wheat was conducted for the first time, using a framework map based on a mapping population, which was available in the form of 100 recombinant inbred lines (RILs). The data on GPC for QTL mapping was recorded by growing the RILs in five different environments representing three wheat growing locations from Northern India; one of these locations was repeated for 3 years. Distribution of GPC values followed normal distributions in all the environments, which could be explained by significant g × e interactions observed through analyses of variances, which also gave significant effects due to genotypes and environments. Thirteen (13) QTLs were identified in individual environments following three methods (single-marker analysis or SMA, simple interval mapping or SIM and composite interval mapping or CIM) and using LOD scores that ranged from 2.5 to 6.5. Threshold LOD scores (ranging from 3.05 to 3.57), worked out and used in each case, however, detected only seven of the above 13 QTLs. Only four (*QGpc.ccsu-2B.1*; *QGpc.ccsu-2D.1*; *QGpc.ccsu-3D.1* and *QGpc.ccsu-7A.1*) of these QTLs were identified either in more than one location or following one more method other than CIM; another QTL (*QGpc.ccsu-3D.2*), which was identified using means for all the environments, was also considered to be important. These five QTLs have been recommended for marker-assisted selection (MAS). The QTLs identified as above were also validated using ten NILs derived from three crosses. Five of the ten NILs possessed 38 introgressed segments from 16 chromosomes and carried 42 of the 173 markers that were mapped. All the seven QTLs were associated with one or more of the markers carried by the above introgressed segments, thus validating the corresponding markers. More markers associated with many more QTLs to be identified should become available in the future by effective MAS for GPC improvement.

Keywords QTL · Bread wheat · Grain protein content · SSR · Composite interval mapping · NIL

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Introduction

Grain protein content (GPC) is an important grain quality trait in bread wheat. The genes/QTLs for this trait are believed to be distributed on at least a dozen chromosomes in tetraploid and hexaploid wheats (Kuspira and Unrau 1957; Law et al. 1978; Morris et al. 1978; Levy and Feldman 1989; Joppa and Cantrell 1990; Stein et al. 1992; Snape et al. 1995; Blanco et al. 1996; Joppa et al. 1997; Mesfin et al. 1999; Prasad et al. 1999; Dholakia et al. 2001; Harjit-Singh et al. 2001). The improvement in GPC and alteration in composition of storage proteins in bread wheat have been a major concern for plant breeders. The selection for high GPC or for a high proportion of some individual essential amino acids/protein subunits, however, is expensive and time-consuming. The development of molecular markers for marker-aided indirect selection for GPC should be a convenient alternative. This indirect marker-aided selection (MAS) is more important for traits like GPC, which exhibit high genotype-environment (g × e) interaction leading to low heritability. In recent studies on tetraploid and hexaploid wheats, a few molecular markers associated with GPC have been identified (Blanco et al. 1996; Mesfin et al. 1999; Prasad et al. 1999; Khan et al. 2000; Dholakia et al. 2001; Harjit-Singh et al. 2001). However, a majority of these molecular markers were identified through simple re-

gression or linkage approaches using independence tests. In two of our earlier studies on hexaploid wheat (Prasad et al. 1999; Harjit-Singh et al. 2001), we also used the simple regression approach for identification of two GPC-associated SSR markers that were later subjected to validation studies using three sets of near-isogenic lines (NILs). These approaches of simple regression or linkage studies have some serious limitations that have been widely discussed and improvements suggested (Melchinger 1998). In this connection, QTL interval mapping, which was first described by Lander and Botstein (1989), has been considered a landmark in quantitative genetics and has been utilized in the present study for the mapping of QTLs for GPC. For this purpose, a framework map was prepared using SSR markers and a recombinant inbred line (RIL) mapping population. The framework map thus prepared was used along with data on the GPC of RILs from five different environments: (1) to study the level of genotype × environment interaction for GPC; (2) to determine the precise positions and effects of QTLs for GPC using interval mapping, and (3) to identify and validate the GPC associated markers using near-isogenic lines (NILs) for use in MAS.

Materials and methods

Plant material

(1) Parental genotypes: a total of four genotypes of bread wheat (*Triticum aestivum* L.), namely PH132 and PH133 (with high GPC) and WL711 and HD2329 (with low GPC) were used, two of them for the development of a RIL population and all four for the development of ten NILs. (2) RIL population: a mapping population consisting of 100 RILs was derived from the cross WL711 (low GPC) × PH132 (high GPC), following the single-seed descent (SSD) method. (3) NILs: the following three crosses were used for developing NILs where, in each case, the parent with high GPC was used as the donor parent (DP), and that with low GPC was used as a recipient parent (RP): (i) WL711 (low GPC) × PH132 (high GPC), (ii) WL711 (low GPC) × PH133 (high GPC), and (iii) HD2329 (low GPC) × PH132 (high GPC). Each of the three F_1s was backcrossed to the respective RP up to BC_6, exercising phenotypic selection for high GPC in each generation until homozygosity was achieved. Thus three high GPC NILs each from WL711 × PH132 and WL711 × PH133 and four high GPC NILs from HD2329 × PH132 were derived.

Evaluation of parents, RIL population and NILs for GPC

The parents along with the RIL population were evaluated at three locations including Ludhiana (location 1) for 3 years, i.e. 1997 (environment I), 1998 (environment II) and 2001 (environment III), and at Meerut (location 2; environment IV) and Pantnagar (location 3; environment V) during 2001. The NILs were evaluated in replicated plots in environment I. The parents and RIL population were raised in replicated plots in all five environments. In all the experiments, each plot consisted of a single row of 2 m with a plot-to-plot distance of 23 cm. The GPC (%) from individual plots was directly obtained using the Infratech Grain Analyser.

DNA isolation and selection of microsatellite markers

DNA was extracted from the leaves of individual plants from all parents, NILs and each of the 100 RILs grown in the field using a modified CTAB method (Weising et al. 1995). The primer pairs for mapped gwm and gdm markers (Röder et al.1998; Pestsova et al. 2000; Röder et al., unpublished) and the new wmc (wheat microsatellite consortium) markers showing polymorphism between the parents of the RIL population were selected for constructing a framework map that was subsequently used for QTL analysis.

PCR, fragment analysis and genetic mapping

For gwm and gdm markers, the PCRs and gel electrophoresis were performed at IPK, Gatersleben (Germany), following Röder et al. (1998), and the fragment sizes were calculated using the computer program Fragment Analyser v. 1.02 (Pharmacia) by comparison with internal size standards. For wmc markers the PCRs and gel electrophoresis were carried out at CCS University, Meerut (India), following Prasad et al. (1999). The markers were mapped using MAPMAKER v. 2.0 program (Lander et al. 1987) based on Kosambi's mapping function (1944) and using a minimum LOD score of 3.0 and maximum recombination frequency of 50%.

Means and frequency distribution for GPC

The means and frequency distributions for GPC of RILs were obtained using the analysis tools of Microsoft Excel. Histograms for GPC of RILs were also prepared using the same tools.

Genotype × environment interaction and rank correlation coefficient analysis

For a study of genotype × environment interaction, analysis of variance was conducted by PROC GLM in SAS (SAS 1996), using genotypes as fixed effects and all the other sources of variation as random effects. Spearman's rank correlations for GPC of RILs in five different environments were also calculated using the SPSS programme.

QTL analysis

The positions and effects of QTLs for GPC in each of the five environments as well as over all the five environments were determined following single-marker analysis (SMA), simple interval mapping (SIM) and composite interval mapping (CIM) using QTL Cartographer v. 1.21 (Basten et al. 1994, 2000; Wang et al. 2001). A LOD score≥2.5 was used to detect a QTL. QTL Cartographer's Zmap QTL, Model 6 with a window size of 10 cM was used for CIM. The number of markers for the background control was set to 5. The significant threshold LOD scores for detection of QTLs were calculated based on 1,000 permutations at $P \leq 0.05$ (Churchill and Doerge 1994; Doerge and Churchill 1996).

The proportion of observed phenotypic variation explained (PVE) due to a particular QTL was estimated by the coefficient of determination (R^2) from the corresponding linear model (single-marker) analysis, and using maximum likelihood for SIM and CIM (Basten et al. 1997).

For each marker that was closest to a QTL, the RILs were grouped into two classes belonging to the two parental alleles of this marker locus. The GPC means of two allele classes were compared using a *t*-test both for significance and for identification of the parent to which the allele having a positive effect on GPC belonged.

Marker validation and detection of introgressed segments from the DP in the NILs

The validation of markers showing association with QTLs for GPC and the detection of chromosomal segments introgressed from the DPs into individual NILs was carried out by genotyping RP, DP and NIL for each mapped marker.

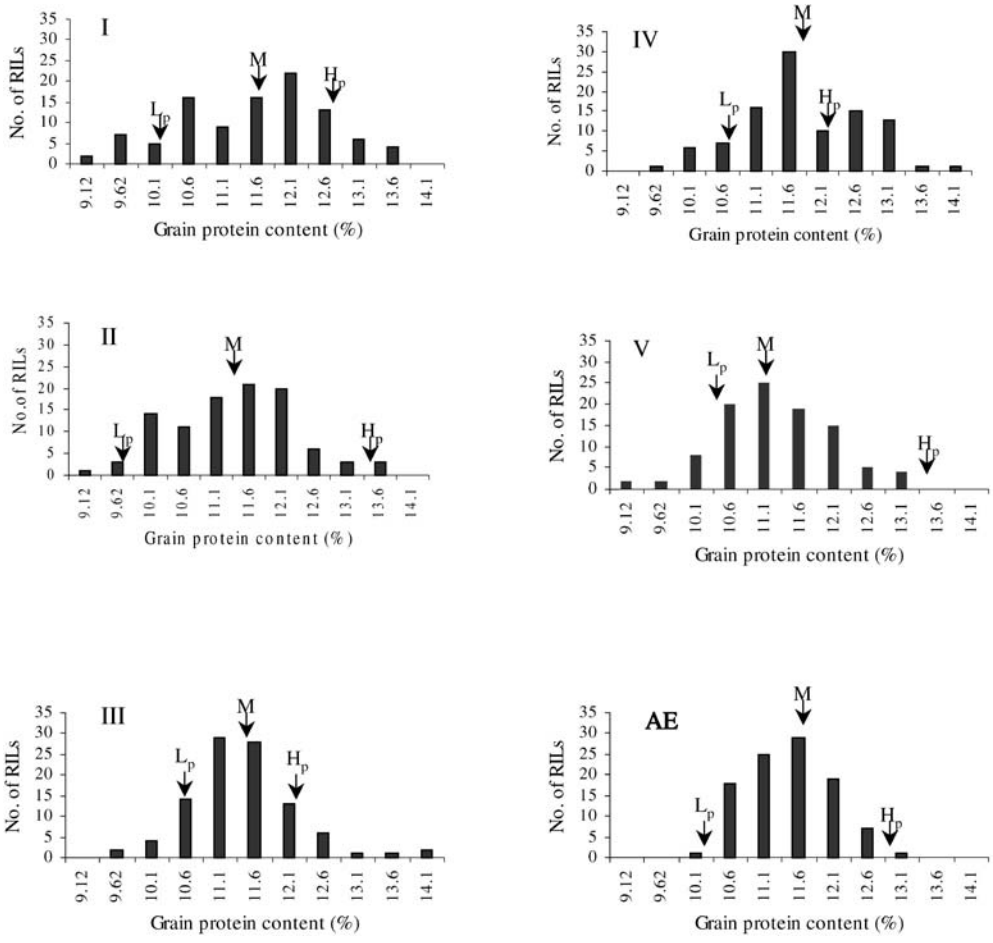

Fig. 1 Frequency distribution of the mean GPC of 100 RILs in five (I to V) different environments and the mean (AE) environment. The mean GPC of WL711 (Lp), PH132 (Hp), and of the RIL population (M), are indicated by *arrows*

Results

Mean GPC of the parents and the RILs

The distribution of GPC among RILs and the values of the mean GPC for each parent and the RILs in different environments are presented in Fig. 1. The mean GPC of the two parents of the RIL population, i.e. WL711 and PH132, differed significantly, and in different environments ranged from 9.7% to 10.83% for WL711 and 12.27% to 13.51% for PH132. The mean GPC of the whole RIL population in different environments ranged from 11.25% to 11.77% and the mean GPC of individual RILs in different environments ranged from 8.76% to 14.17%. The GPC of individual RILs showed a good fit to normal distribution, and transgressed the GPC of the low protein parent (WL711) in all the environments and that of the high protein parent (PH132) in four of the five environments.

Analysis of variance for GPC and correlation between ranks of RILs in different environments

The results of analysis of variance of GPC in the RIL population are given in Table 1. The mean squares due to RILs, environments and RILs × environment interaction were significant. All possible rank correlation coefficients between the ranks of RILs, based on GPC, in different environments were also positive and significant (Table 2).

Table 1 Analysis of variance for grain protein content (GPC)

Source of variation	Degrees of freedom	Mean square
Environments	4	14.61***
Replications (Environments)	10	0.74
RILs	99	4.91***
RILs × environments	396	1.72***
Pooled error	990	0.37

***$P < 0.001$

Table 2 Rank correlation coefficients between grain protein content (GPC) in five environments

Environments	I	II	III	IV
II	0.463**			
III	0.532**	0.414**		
IV	0.312**	0.224*	0.217*	
V	0.264**	0.288**	0.255*	0.429**

*$P < 0.05$; **$P < 0.01$

Table 3 Putative QTLs detected using three approaches in an RIL population from a WL711 × PH132 cross

QTL	Chromosome arm	Closest marker	Environment[a]	SMA		SIM		CIM		Allele for positive effect
				LOD	R^2 (%)	LOD	R^2 (%)	LOD	R^2(%)	
QGpc.ccsu-2A.1	2AS	*Xgwm830*	IV	–	–	–	–	3.9	20.75	WL711
QGpc.ccsu-2A.2	2AS	*Xgwm726*	II	–	–	2.6	10.43	–	–	–
QGpc.ccsu-2B.1	2BL	*Xgwm1249*	V	2.9	5.74	–	–	3.5	13.39	PH132
QGpc.ccsu-2D.1	2DL	*Xgwm1264*	I	3.91	16.50	3.7	16.01	5.5	19.60	PH132
	2DL	*Xgwm1264*	II	3.67	15.56	3.8	16.93	3.9	11.48	PH132
	2DL	*Xgwm1264*	III	–	–	–	–	3.0	10.36	PH132
	2DL	*Xgwm1264*	AE	3.40	14.48	3.3	16.31	4.1	13.91	PH132
QGpc.ccsu-2D.2	2DL	*Xgwm1204*	I	–	–	–	–	5.6	2.95	PH132
QGpc.ccsu-3D.1	3DS	*Xgwm456*	III	–	–	2.6	16.41	4.0	16.27	PH132
QGpc.ccsu-3D.2	3DS	*Xgwm892*	AE	–	–	–	–	3.6	13.99	PH132
QGpc.ccsu-4A.1	4AL	*Xgwm894*	V	–	–	–	–	2.7	13.36	–
QGpc.ccsu-4A.2	4AL	*Xgwm397*	IV	–	–	–	–	2.9	8.21	WL711
QGpc.ccsu-6B.1	6BS	*Xgwm133*	AE	–	–	–	–	2.5	16.38	–
QGpc.ccsu-6B.2	6BS	*Xgwm889*	I	3.3	14.98	–	–	–	–	WL711
QGpc.ccsu-7A.1	7AS	*Xgwm1171*	II	2.85	0.63	3.6	35.80	6.5	32.44	PH132
QGpc.ccsu-7D.1	7DS	*Xgdm86*	I	4.04	9.34	3.2	15.84	–	–	WL711

[a] Environment I = Ludhiana 1997; II = Ludhiana 1998; III = Ludhiana 2001; IV = Meerut 2001; V = Pantnagar 2001; AE = across environment

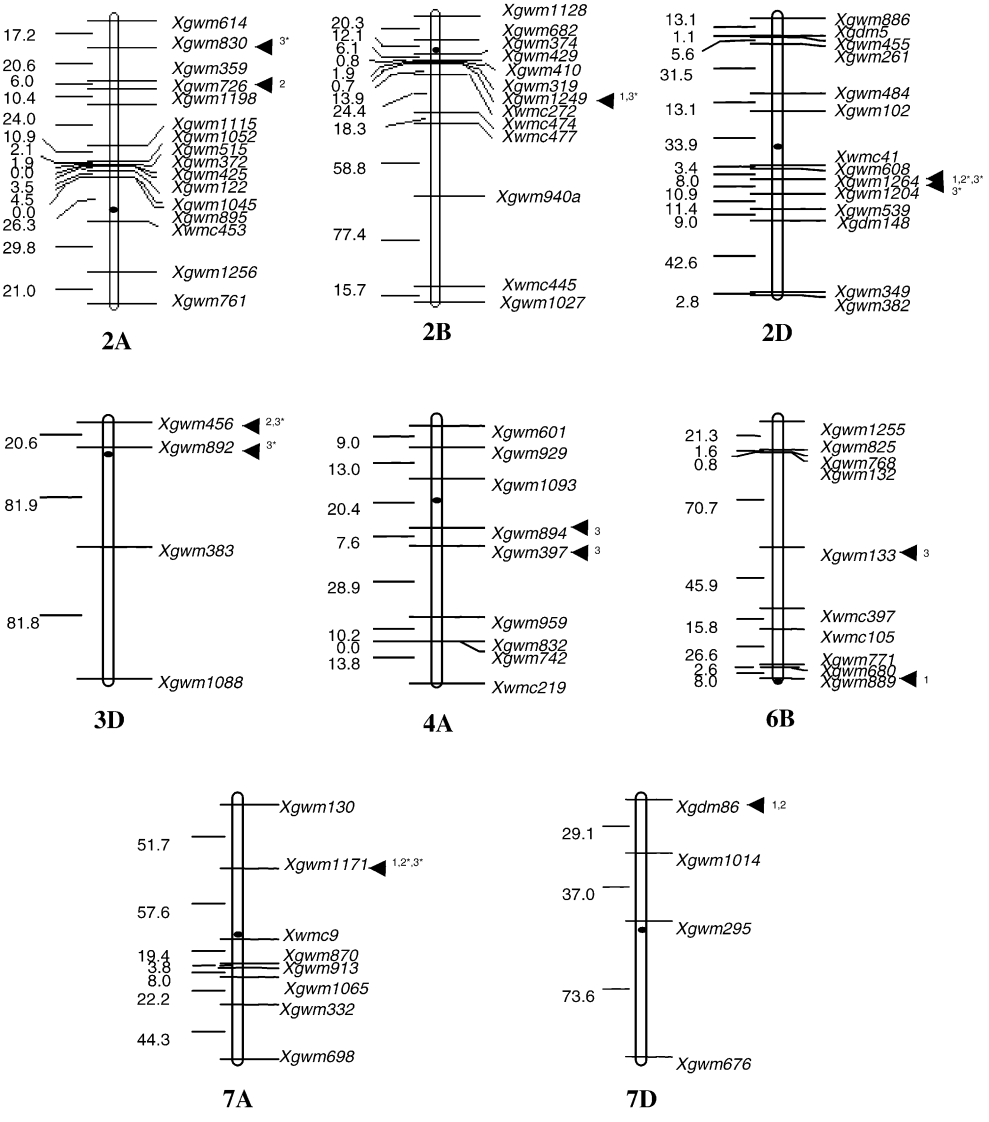

Fig. 2 Map location of putative QTLs for GPC detected in this study. Short arms of the chromosomes are at the top and the chromosome numbers are indicated at the bottom. Centromeres are indicated by a dark oval. Mapped SSR markers are indicated to the right and their corresponding genetic distances are indicated to the left. *Arrowheads* indicate the positions of putative QTLs; 1, 2 and 3 indicate that QTL is detected by SMA, SIM and CIM, respectively, and the asterisk (*) indicates that the QTL was detected above the threshold LOD score

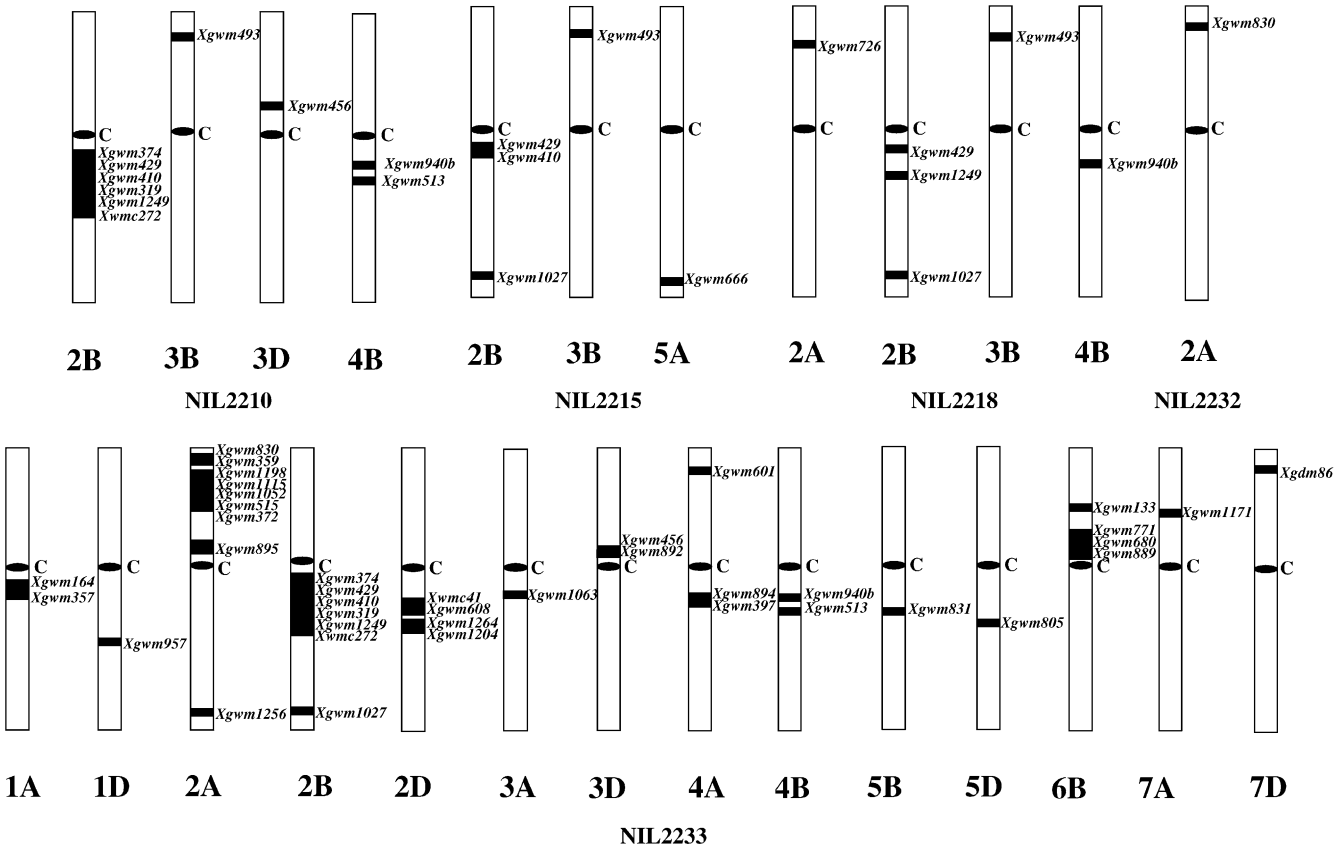

Fig. 3 Validation of linked SSR markers for GPC in NILs. Short arms of chromosomes are at the top. The chromosome number and the identities of NILs are given at the bottom. The centromeres are indicated by a solid ellipse labeled *C*. The introgressed segments from the donor parents are represented by a *solid rectangle* and the identity of SSR markers located in the introgressed segments are indicated to the right of the chromosomes

Preparation of a framework linkage map

A total of 509 SSR primer pairs, comprising 350 gwm/gdm primers (Röder et al. 1998; Pestsova et al. 2000; Röder et al., unpublished) and 159 wmc (Wheat Microsatellite Consortium) primers, were used for detection of polymorphism between the two parent genotypes (WL711 and PH132) of the RIL mapping population. Out of all the primers detecting polymorphism, 171 primer pairs were subsequently used for genotyping all the 100 RILs. Using this information, 173 loci were finally mapped on all the 21 different chromosomes covering a total of 3,272.4 cM. The number of markers on individual linkage groups varied from four (1D, 3D, 4D, 6D and 7D) to as many as 16 (2A). Only two markers (*Xgwm940* and *Xwmc415*) were mapped on two loci each. SSRs were more abundant on the B genome (74) than either on the A (60) or D (39) genome.

QTL analysis for GPC

The results of QTL analysis are presented in Table 3 and Fig. 2. The threshold LOD scores for SIM were 3.48, 3.19, 3.05, 3.20, 3.23 and 3.24 for environments I, II, III, IV, V and over all the environments. Similarly, the threshold LOD scores for CIM were 3.55, 3.51, 3.21, 3.42, 3.40 and 3.57 for environments I, II, III, IV, V and over all the environments. At a LOD ≥ 2.5, a total of 13 QTLs for GPC, spread on eight different chromosomes (2A, 2B, 2D, 3D, 4A, 6B, 7A and 7D) were detected following three different methods (SMA, SIM and CIM) of QTL analysis. Two of these QTLs were detected by all the three methods, three were detected by two of the three methods and eight QTLs were detected by only one of the three methods including six that were detected by CIM and one each detected by SMA and SIM. In CIM, seven QTLs (*QGpc.ccsu-2A.1 QGpc.ccsu-2B.1 QGpc.ccsu-2D.1*, *QGpc.ccsu-2D.2*, *QGpc.ccsu-3D.1*, *QGpc.ccsu-3D.2* and *QGpc.ccsu-7A.1*) out of ten QTLs that were detected using LOD ≥ 2.5 were also available at/or above the threshold LOD score. Two of these seven QTLs were also detected using SIM. The phenotypic variation explained (PVE) by individual QTLs detected ranged from 0.63% to 16.50% in SMA, 10.43% to 35.80% in SIM and 2.95% to 32.44% in CIM. Differences between mean values of GPC worked out for RILs belonging to two classes having alternative alleles of the closest marker for a QTL were significant in only ten of the above 13 QTLs. For these ten QTLs the high protein alleles were contributed by WL711 in four cases and by PH132 in six cases. It may be noted that the three QTLs for which the associated marker allele classes did not show significant differences for GPC were those which

were detected at a LOD score ≥ 2.5, that was lower than their corresponding thresholds.

Detection of donor chromosome segments and validation of markers linked with QTLs for GPC in NILs

All SSR markers from the framework map used for analysis of QTLs for GPC were tried on ten NILs (1) to locate the segments introgressed from the DPs and (2) to confirm the utility of QTL-linked SSR markers in marker-aided selection (MAS) in different genetic backgrounds for improvement in GPC. The details of the intro-gressed segments from DPs in NILs are shown in Fig. 3. In five of these ten NILs, 38 segments from 16 chromosomes carried 42 of the 173 mapped SSR loci from their corresponding DPs (Fig. 3). These 42 SSRs included markers associated with all the seven QTLs in one NIL and two markers associated with two of the seven QTLs in one NIL, and one marker each associated with each of the two different QTLs in two NILs. In the remaining one NIL (NIL2215), none of the introgressed segments carried markers associated with any of the seven QTLs.

Discussion

Genotype × environment interaction and efficiency of CIM for QTL analysis

QTL interval mapping is a significant development in quantitative genetics. However, very few studies using this approach have been conducted in bread wheat, particularly for a study of the genetics of grain protein content (GPC). Keeping this in view, the present study was conducted to identify QTLs for this important trait and to determine locations and effects of these QTLs using QTL interval mapping. It may be recalled that data were recorded on the mapping population grown in five different environments, and three different methods were used for QTL analysis. Normal distributions of GPC values observed in each of the five environments during the present study can be attributed to significant g × e interactions (Table 1) and/or a number of unidentified QTLs with minor effects, since only 2–4 QTLs could be detected in each of the five environments. However, the g × e interaction in the present study seems to be predictable and involved a similar response by all the RILs in individual environments as obvious from significant rank correlations (Table 2).

The total number of QTLs detected in all the five environments and using three different methods (SMA, SIM, CIM), however, were as many as 13. It may be recalled that SMA and SIM detected five QTLs each, while CIM detected ten QTLs (Table 3); a higher number of QTLs detected by CIM may be attributed to elimination of the background effect that leads to confounding of the results in SMA and SIM. Only two QTLs were detected by each of the three methods, and only one of these two QTLs was detected in more than one environment (in three of the five environments). There were two additional QTLs that were detected by two of the three methods employed. All other QTLs were detected each in a solitary environment.

The shortcomings of SMA and SIM are now well known. They are believed to have a bias *firstly*, due to the presence of multiple linked QTLs and *secondly,* due to the reduced power of QTL detection attributed to inflation of phenotypic variance within a marker allele class due to segregation of unlinked QTLs (Knott and Haley 1992). The CIM, on the other hand, combines maximum likelihood interval mapping with multiple regression using marker cofactors, so that it largely overcomes the above problems associated with SMA and SIM, and detects the position and effect of a QTL more precisely (Jansen and Stam 1994; Zeng 1994). Earlier reports suggested that, using SMA, the proportion of false positives among the QTLs can be much larger than the nominal Type-I error would allow (Basten et al. 1994). This explains why only two of the ten QTLs detected by CIM, were also detected by SMA and SIM; the other QTLs detected by SMA/SIM presumably being false positives. Since CIM is considered to be relatively more efficient, a higher level of confidence can be placed on QTLs detected by this method. Therefore, several QTLs detected by the CIM but not by SMA and SIM may represent false negatives in both SMA and SIM. However, even by CIM approach, none of the QTLs was detected in each of the five environments, although there was one QTL that was detected in three of the five environments. This may also mean that even in the CIM approach, false negatives can be available in each of the five environments, due to possible QTL × environment interaction as indicated by significant g × e interactions.

It may also be noted that the LOD score, used for detection of the above ten QTLs by CIM was 2.5 or above. However, only seven of these QTLs were detected at a LOD score equal to or above the threshold values, which ranged from 3.21 to 3.57 in the CIM approach using data from five environments. The above seven QTLs also included the two QTLs that were detected by all the three methods, and another two QTLs which were detected by only two of the three methods. Furthermore, the three QTLs detected at a LOD score of 2.5 or above, but at a LOD score lower than the threshold, were each detected either in a single environment or by using the data pooled over the environments. These three QTLs were not detected by SMA or SIM. Therefore the possibility of these QTLs being false positives cannot be entirely ruled out. The above seven QTLs were located on five different chromosomes, i.e. 2A, 2B, 2D, 3D and 7A. QTLs/genes for GPC on all these five chromosomes were also reported in earlier studies on bread and tetraploid wheats (Kuspira and Unrau 1957; Levy and Feldman 1989; Dholakia et al. 2001; Harjit-Singh et al. 2001).

Three QTLs that were not available in CIM, including one each detected by SMA or SIM alone and one QTL detected by both SMA and SIM may also be considered as false positives, although their being false negatives in CIM can not be ruled out. Therefore, the present study brings out clearly the superiority of CIM over SMA and SIM, and also the desirability of using a threshold value worked out for individual experiments on QTL mapping.

QTL effects

It may also be noted that the seven QTLs detected by CIM using threshold LOD scores varied in QTL effects, measured as the phenotypic variation explained (PVE) which ranged from 2.95% to 32.44%. As discussed earlier, the most important of the above seven QTLs was *QGpc.ccsu-2D.1*, for which PVE ranged from 11% to 20%. Another QTL (*QGpc.ccsu-7A.1*) having a large effect in only one environment (Environment II, i.e. Ludhiana 1998) had 32.44% PVE; its effect in other environments is presumably masked due to QTL × environment interaction. Four of the remaining five QTLs detected by CIM at a threshold LOD score also deserve attention, because they explain as much as 13% to 21% of the phenotypic variation. Only one remaining QTL had a very low effect accounting for only about 3% variation for GPC. Although the QTLs detected in the present study and the ones detected in a study on tetraploid wheat by Blanco et al. (1996) were different, the phenotypic variation explained by individual loci for GPC was comparable. In contrast to these results of QTL effects, the genetic studies had suggested that the parents of the RILs used in the present study differed by a major gene for GPC (Dhaliwal et al. 1994). Similarly, a major QTL accounting for up to 66% of variation in GPC was located on a small segment of 6B in a derived tetraploid wheat, RSL#68 (Joppa et al. 1997). However, no such major gene/QTL on chromosome 6B of bread wheat has been reported so far, although several other chromosomes (1A, 1B, 3D, 4D, 5A, 5B, 5D, 7A and 7B) of hexaploid wheat have been found to influence GPC (Kuspira and Unrau 1957; Law et al. 1978; Morris et al. 1978; Stein et al. 1992; Snape et al. 1995).

Marker-trait association

During the present study, the molecular markers closest to each of the seven QTLs identified at the threshold LOD score of CIM also displayed significant marker-trait association.The high GPC alleles for six of these seven QTLs belonged to PH132, the parent with high GPC, and the high GPC allele for the remaining solitary QTL (*QGpc.ccsu-2A.1*) belonged to WL711, the parent with low GPC. Therefore, the QTLs for high GPC appear to be distributed among the parents of the RIL population and may account for the observed transgressive segregation for GPC in the RIL population.

QTLs, markers and MAS

It has been argued that the most conservative approach to identify QTLs useful for MAS is to consider those QTLs that are detected by at least two of the three methods of analysis and/or are detected in multiple environments or in the mean environment (Veldboom and Lee 1996; Fulton et al. 1997; Moncada et al. 2001). Therefore, the four QTLs (*QGpc.ccsu-2B.1*, *QGpc.ccsu-2D.1*, *QGpc.ccsu-3D.1*, *QGpc.ccsu-7A.1*) detected by CIM and by at least one more method, and another QTL (*QGpc.ccsu-3D.2*) that was identified in the mean environment, may represent important QTLs during MAS aimed at improvement in the GPC of bread wheat. From the above five QTLs, the most important QTL (*QGpc.ccsu.2D.1*) detected in this study is, however, the one, which was detected in three environments by CIM; in two of these three environments it was also detected by SMA and SIM. Since, this QTL is the same, which was detected by us earlier using SMA, the present study once again validated the marker *Xwmc41* for this QTL for the GPC located on 2DL. However, the present study allowed identification of another marker (*Xgwm1264*), which is relatively closer to the above QTL and can be used for MAS more effectively than *wmc41* earlier identified by us (Prasad et al. 1999). Among the above five QTLs, another important QTL for GPC is *QGpc.ccsu-7A.1* (environment II = Ludhiana 1998), which was detected by all three methods in the same environment. The two additional QTLs that were detected by more than one method could also be useful for MAS. In addition to the above five QTLs, two more putative QTLs (*QGpc.ccsu-2A.1* and *QGpc.ccsu-2D.2*) identified through CIM using a threshold LOD score were detected in only a single environment each.

Validation of markers using NILs

The validation of markers associated with the seven putative QTLs for GPC was also carried out using three sets of ten NILs for GPC derived from three different crosses involving two different DPs, each with a high GPC (Harjit-Singh et al. 2001). It may be recalled that in five of the ten NILs (derived from two different crosses), 38 segments in 16 chromosomes carried 42 of the 173 mapped SSR loci, for which marker alleles matched those of the DP (Fig. 3). These 42 SSRs included markers associated with all the seven QTLs, although a number of these introgressed segments were not associated with any of the QTLs identified during the present study. Further, in one (NIL 2215) of the above five NILs, the segments transferred from the DP carried markers that were not associated with any of the seven QTLs identified during the present study. It may also be noted that all the four (2210, 2218, 2232 and 2233) of the above five NILs, each carrying one or more QTL-associated markers, were derived from crosses other than the cross involved in developing the RILs used for QTL analysis.

Table 4 Mean grain protein content in parental genotypes and the near-isogenic lines (NILs), showing QTLs for GPC introgressed into NILs (modified from Harjit-Singh et al. 2001)

Parents and NILs	GPC (% dry weight)	QTLs introgressed into NILs	
		Number	Name
(a) Parents			
W711 (RP)	9.8		
PH 132 (DP)	13.8		
NILs			
2147	11.6	–	–
2149	11.6	–	–
2150	11.2	–	–
(b) Parents			
WL711 (RP)	9.8		
PH133 (DP)	12.4		
NILs			
2210	12.4	2	*QGpc.ccsu-2B.1*, *QGpc.ccsu-3D.1*
2215	11.9	–	–
2218	11.9	1	*QGpc.ccsu-2B.1*
(c) Parents			
HD2329 (RP)	9.7		
PH132 (DP)	13.8		
NILs			
2230	12.2	–	
2231	11.4	–	
2232	11.9	1	*QGpc.ccsu-2A.1*
2233	11.7	7	*QGpc.ccsu-2A.1, QGpc.ccsu-2B.1, QGpc.ccsu-2D.1, QGpc.ccsu-2D.2, QGpc.ccsu-3D.1, QGpc.ccsu-3D.2, QGpc.ccsu-7A.1*

Two of the above four NILs (2232 and 2233) were derived using PH132 as DPs, which was also one of the parents of the mapping population used for QTL analysis. In these two NILs (particularly the NIL 2233), all the seven QTLs seem to be introgressed, since the QTL-associated SSR alleles in these NILs were the same as those in PH132 (Table 4; Fig. 3). The remaining two NILs, i.e. NIL2210 and NIL2218, having segments of chromosomes carrying QTL-associated marker(s), were derived from a cross in which the DP was PH133, which was not a parent of the mapping population. Of these two NILs, NIL2210 had two QTL-associated markers (*gwm1249, gwm456*), one of these (*gwm1249*) being also present in NIL2218. The allele for *gwm1249* was a null allele in PH132 as well as in PH133 and its two corresponding NILs (2210, 2218). However the allele for the other marker, *gwm456*, differed in length in PH132 and PH133, although different alleles at the same locus in the two DPs could still be associated with the same QTL allele contributing to the high GPC.

The four NILs, which validated the markers, had a GPC that was 20.62% to 26.56% higher than their corresponding RPs, i.e. WL711 and HD2329 (Table 4). This increase of GPC in NILs over their corresponding RPs, however, represented as much as 48.78% (NIL2233) to 100% (NIL2210) of the excess GPC in DP over RP. However, the four different NILs did not have the same pattern of relationship between the number of QTL-associated markers and the level of increase in GPC. For example, in NIL2233 although the markers associated with each of the seven QTLs were validated, yet the improved GPC (11.7%) of this NIL represented only 48.78% of the excess GPC of the DP over that of the corresponding RP. In contrast, in NIL2210, although the GPC was as high as 12.4%, representing 100% of the excess GPC of the DP, yet only two of the seven marker-associated QTLs were validated in this NIL. This situation in NIL2210, relative to NIL2233, is indeed intriguing and could be due to introgression of additional QTLs, not identified during the present study. Therefore, it appears that there may be more QTLs other than the ones identified during the present study for which markers need to be developed. In any case, we believe that the seven QTL-associated markers for GPC identified during the present study, along with the new markers that may be identified in the future, would be successfully used by MAS for an improvement in GPC and eventually grain quality in bread wheat.

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