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Physical map of the wheat high-grain protein content gene *Gpc-B1* and development of a high-throughput molecular marker

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Summary

• Grain protein content (GPC) is important for human nutrition and has a strong influence on pasta and bread quality. A quantitative trait locus, derived from a *Triticum turgidum* ssp. *dicoccoides* accession (DIC), with an average increase in GPC of 14 g kg^{-1} was mapped on chromosome 6BS.

• Using the wheat–rice colinearity, a high-density map of the wheat region was developed and the quantitative trait locus was mapped as a simple Mendelian locus designated *Gpc-B1*. A physical map of approx. 250 kb of the *Gpc-B1* region was developed using a tetraploid wheat bacterial artificial chromosome library.

• The constructed physical map included the two *Gpc-B1* flanking markers and one potential candidate gene from the colinear rice region completely linked to *Gpc-B1*. The relationship between physical and genetic distances and the feasibility of isolating genes by positional cloning in wheat are discussed.

• A high-throughput codominant marker, *Xuhw89*, was developed. A 4-bp deletion present in the DIC allele was absent in a collection of 117 cultivated tetraploid and hexaploid wheat germplasm, suggesting that this marker will be useful to incorporate the high GPC allele from the DIC accession studied here into commercial wheat varieties.

Key words: colinearity, grain protein content, physical map, quantitative trait loci (QTL), rice, wheat.

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Introduction

Grain protein content (GPC) affects quality of pasta and bread and is important for human nutrition. A potential approach to increase GPC in wheat is to exploit the high GPC genes from wheat wild relatives. Wild emmer, *Triticum turgidum* L. ssp. *dicoccoides*, contains higher GPC (170–273 g kg^{-1}) than most of the bread wheat cultivars (110–170 g kg^{-1}) and therefore, may be a promising source of as yet untapped high GPC genes (Gerechter-Amitai & Grama, 1977; Avivi, 1978; Grama *et al.*, 1983; Levy & Feldman, 1988; Nevo *et al.*, 2002).

A promising source of high GPC was detected in a survey of wild wheat (accession FA–15-3; Avivi, 1978), referred to as dicoccoides or DIC hereafter. Joppa & Cantrell (1990) developed a complete set of disomic substitution lines from each of the chromosomes of this dicoccoides accession into the tetraploid durum cultivar 'Langdon' (LDN). These chromosome substitution lines can be used to facilitate the precise mapping of quantitative trait loci (QTL). In these populations, differences between the parental lines are restricted to a single chromosome, decreasing the genetic variability and increasing the sensitivity of the QTL analysis. This strategy has been used for more than half a century to analyze complex characters in wheat (Kuspira & Unrau, 1957; Sears, 1953; Law, 1967).

The substitution of the complete chromosome 6B of DIC into LDN, LDN(DIC-6B), showed the highest protein concentration. This significant increase in GPC was associated with an increase in the quality of the pasta products (Joppa *et al.*, 1991) and was not associated with a significant decrease in grain yield or kernel weight (Cantrell & Joppa, 1991).

When introduced into adapted durum germplasm, the DIC allele was also associated with an increase in protein content (15 g kg^{-1}) and nonsignificant effects on protein quality and plant height, although a negative correlation between yield and GPC was observed in one of the three locations tested (Chee *et al.*, 2001). The introgression of the DIC allele into common wheat varieties also resulted in higher amounts of GPC (Mesfin *et al.*, 1999; Khan *et al.*, 2000). In addition, wheat lines carrying the DIC allele exhibit higher levels of soluble proteins and amino acids in flag leaves after anthesis compared with lines carrying the LDN allele (Kade *et al.*, 2005).

Other QTL studies suggested that a major gene affecting GPC in the *Triticeae* was probably located in the short arms of the chromosomes from homoeologous group 6. Blanco *et al.* (2002) identified two quantitative trait loci (QTL) in tetraploid wheat, *QPro.mgb-6A1* and *QPro.mgb-6B*, located on the distal regions of chromosomes 6AS and 6BS, respectively. A significant quantitative trait locus for GPC on chromosome arm 6AS in hexaploid wheat was associated with amplified fragment length polymorphism (AFLP) marker $XE38M60_{200}$ (Sourdille *et al.*, 2003). Finally, a major quantitative trait locus for high-GPC near marker *Hvm74* was reported in barley chromosome 6HS (See *et al.*, 2002).

The evaluation of a mapping population of recombinant substitution lines (RSLs) from the cross between LDN(DIC-6B) and LDN showed that the increase in GPC in LDN(DIC-6B) was associated with a QTL located on the short arm of chromosome 6B within the *Xmwg79–Xabg387* interval (Joppa *et al.*, 1997). More recently, using field trials with 10 replications and a large set of secondary RSLs from the cross between LDN and RSL65, the source of GPC variation was mapped as a single Mendelian locus within a 2.7-cM region encompassed by restriction fragment length polymorphism (RFLP) loci *Xcdo365* and *Xucw67*. This locus was designated *Gpc-B1* (Olmos *et al.*, 2003).

To further delimit the location of *Gpc-B1*, we characterized the microcolinearity between rice and wheat in this region and used the rice genome sequence as a stepping stone for the development of new markers in the region from the corresponding wheat expressed sequence tags (ESTs). This approach enabled us to narrow down the position of the *Gpc-B1* locus to a 0.3-cM region flanked by polymerase chain reaction (PCR) markers *Xucw79* and *Xucw71*, which corresponds to a 64-kb region in rice chromosome 2 (Nipponbare BAC (bacterial artificial chromosome) AP004061; Distelfeld *et al.*, 2004). This region is currently represented in the sequenced rice genome by BAC AP005647.

Genetic improvement of GPC has been a frequent target of bread and pasta wheat breeding programmes because of the potential economic benefit of reducing the necessity for expensive nitrogen (N) fertilization. Conventional breeding, however, has made slow progress in the improvement of this trait because of the complex genetic system governing GPC, the strong influence of the environment, and the negative correlation between GPC and grain yield (Simmonds, 1995). Efforts to improve GPC without selecting for low yield can be accelerated by the identification of the genes that affect GPC and the direct selection of positive alleles with molecular markers.

The specific objectives of this study were: (1) to further explore the microcolinearity between the 64-kb region in rice and the orthologous 0.3-cM region in wheat containing the *Gpc-B1* locus; (2) to construct a physical map of the 6BS chromosome arm region including the *Gpc-B1* gene; and (3) to develop a tightly linked codominant PCR marker for *Gpc-B1*, which could eliminate the use of a restriction enzyme digestion step that is required in the current cleavage amplified polymorphic sequence (CAPS) markers, and that shows polymorphisms within a large set of commercial wheat varieties.

Materials and Methods

Mapping population

The mapping population included 85 homozygous RSLs developed by L. R. Joppa from the cross LDN(DIC-6B) × LDN (Joppa *et al.*, 1991), 134 F_2 plants from the cross RSL65 × LDN used in the mapping of the GPC gene within the *Xcdo365* and *Xucw67* interval (Olmos *et al.*, 2003) and 291 new F_2 plants from the same cross (a total of 935 gametes). The recombinant plants from the first two populations were previously evaluated for GPC in three field experiments (Olmos *et al.*, 2003), but the new recombinant lines have not yet been characterized for GPC. All 935 gametes were used in the present wheat–rice colinearity study. Markers developed at the University of California, Davis, CA, USA, are identified by the three letter designator '*ucw*' whereas those developed at the University of Haifa, Israel, use the '*uhw*' laboratory designator according to the rules for the Catalogue of Gene Symbols for Wheat (McIntosh *et al.*, 2005).

Hybridization procedures

Plant nuclear DNAs were isolated from leaves of single plants following the procedure described by Dvorak *et al.* (1988). Hybridizations of the BAC library high-density filters and of genomic Southern blots were performed as described by Dubcovsky *et al.* (1994). The genomic Southern blots used for RFLP mapping consisted of DNAs of the parental genotypes LDN(DIC-6B) and LDN digested with 24 different restriction enzymes (*Asc*I, *Apa*I, *Ava*II, *Bam*HI, *Bfa*I, *Bgl*I, *Bst*EII, *Bst*NI, *Dde*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hin*dIII, *Msp*I, *Nco*I, *Nde*I, *Sac*I, *Sau*3AI, *Ssp*I, *Stu*I, *Sty*I and *Xba*I).

Comparative mapping

The rice genes from the 64-kb colinear rice sequence and their *Triticeae* homologues were identified using the following approach. Predicted open reading frames (ORFs) and potential

Table 1 Source and information on restriction fragment length polymorphism (RFLP) probes that were used in the current research

Source	Triticeae EST	TBLASTN *E*-value	Primer sequence	Length (bp)	Locus
Rice gene *OSJNBa0026E05.13*	BE494838 BE401694	$5e^{-45}$	GAAGATGGTGGACGAGGAGA ACAAATCTGTAGCCCGTTGC	N/A	Not mapped
Rice gene *OSJNBa0026E05.15*	BQ609014	e^{-125}	CAACGTCTGCGCCTACAAC TCAACGAAATCGTGCTCATC	550	Not mapped
Rice gene *OSJNBa0026E05.18*	BQ789353	e^{-54}	ACCTCCAAGTGCGTCAGC ACTGCATGCCTGGTGAAGAC	466	*Xuhw83*
Rice gene *OSJNBa0026E05.19–1*	BE444066	$4e^{-43}$	TGATGAGATTTCTCCACAAAAGAA TCTTGATGAACCCGGTTAGAA	750	*Xuhw84*
Rice gene *OSJNBa0026E05.24*	AL826407	$5e^{-37}$	CAGAATGGACCCTTTGTGGT CCTGAAGGCAATAACGTCTCC	506	*Xucw90*
Rice gene *OSJNBa0026E05.30*	BU995216 BQ753500	e^{-118}	CATTTGGCAATGCAACTGAG TCAACCCTTTTAAGCAATTTGAA	1405	*Xucw71*[a]
Wheat BAC 409D13	–	–	CAAGCAATGGTCAGCGTAGA GAGCATTGCTGGTGTTGAGA	966	*Xuhw86a*[b]

EST, expressed sequences tag.
[a]Distelfeld *et al*. (2004).
[b]*Xuhw86a* locus located 2944-bp of locus *Xuhw86* (downstream from the T7-end of BAC 409D13).

exon/intron boundaries of the colinear rice region were obtained from the TIGR automatic annotation (http://www.tigr.org) and Gramene (http://www.gramene.org). The potential identities of predicted coding regions were tested by searches against the nonredundant protein and DNA databases, and against the EST database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLASTN, BLASTX and BLASTP programs. The rice ORFs predicted in BAC AP005647 and the homologous *Triticeae* sequences were used to search (using TBLASTX) the cereal repeats database at TIGR (http://www.tigr.org/tdb/e2k1/osa1/blastsearch.shtml) and the TREP database at Grain Genes (http://wheat.pw.usda.gov/ggpages/Repeats/index.shtml) to eliminate predicted proteins with similarities to repetitive elements.

Physical maps

The 28 high-density filters of a tetraploid wheat BAC library of RSL65, which carries a 30-cM DIC 6BS chromosome segment including the high GPC gene (Cenci *et al.*, 2003), were used for hybridization with probes from the *Gpc-B1* region (Table 1). Positive BAC clones were confirmed by PCR and Southern blots of *Hin*dIII fingerprints. To establish the genome origin of the BAC clones, genome specific primers were tested in nulli-tetrasomic lines N6AT6B and N6BT6A (Sears, 1966). Fingerprinting of the positive BAC clones was performed with the SNapshot labeling kit (Applied Biosystems, Foster City, CA, USA) and capillary electrophoresis on an ABI3730 (ABI PRISM 3730 DNA sequencer; Applied Biosystems) (Luo *et al.*, 2003). The FPC program (Marra *et al.*, 1997) was used to build the initial BAC contigs that were further confirmed by *Hin*dIII fingerprinting and hybridization or sequencing of BAC ends and PCR assays.

BAC-end sequencing

The BAC-end sequencing was preformed with the DNA walking *SpeedUP* kit (See-gene, Seoul, South Korea) according to the manufacturer's instruction. The vector specific primers designed based on the pIndigoBAC536 BAC vector sequence (supplied from the Clemson University Genomics Institute, SC, USA) (Luo *et al.*, 2001) included Outer T7-end 5′-GTTTTCCCAGTCACGACGTT-3′, Inner T7-end 5′-ACGACTCACTATAGGGCGAAT-3′, Outer SP6-end 5′-TGTGGAATTGTGAGCGGATA-3′, and Inner SP6-end 5′-CGCCAAGCTATTTAGGTGACA-3′. These primers were used in combination with the 'DNA Walking ACP (DW-ACP) primer' provided with the kit and designed to capture unknown target sites. Using this method, we were able to amplify fragments in the range of 400–5000 bp. These PCR products were purified from agarose gels, cloned with pGEM-T Easy kit (Promega, Madison, WI, USA) and sequenced.

Results

Identification of low copy-number candidate genes from rice

The 64-kb chromosome segment from rice chromosome 2, which is colinear with the 0.3 cM *Gpc-B1* region in wheat (Distelfeld *et al.*, 2004), includes 11 putative genes, of which four have significant similarities to known transposable elements in the

Table 2 Positive clones from the tetraploid bacterial artificial chromosome (BAC) library for loci *Xucw71*, *Xuhw83*, *Xuhw84* and *Xuhw86a*

Locus	Number of clones	A genome BAC clones	B genome BAC clones
Xucw71	10	154N17, 445P02, 574J23, 797F22, 837O18, 976M3, 1217E11	409D13, 947A13, 1025F04
Xuhw83	8	153M06, 431C01, 678J09, 866B21, 884P09, 1130E11, 1229O05	770E02
Xuhw84	11	8F18, 154 N17, 445P02, 574J23, 797F22, 837O18, 1105M18, 1217E11	409D13, 947A13, 1025F04
Xuhw86a	4	_	57H16, 409D13, 900C08, 916O17

Table 3 Polymerase chain reaction (PCR)-based markers for loci *Xuhw83*, *Xuhw84*, *Xuhw86, Xuhw89* and *Xucw96*

Locus name and marker type	Primer name	Primer sequence	Restriction enzyme	Expected size LDN (bp)
Xuhw83/CAPS	UHW83-BF	CATCACGGGGTGTCTAAGGT	*Hae*III	413
	UHW83-R	GCCAAGAGAGGTTAGGCAAA		
Xuhw84/CAPS	UHW84-BF	CAGGAGGACTACAGGGAAGTCT	*Sty*I	549
	UHW84-R	CGCGGTTCTTCTACCTTGTT		
Xuhw86/dCAPS	UHW86-BF	CAACTTCGATATGCTTTGTCC**A**T[a]	*Nla*III	133 + 24
	UHW86-R	TGCCAATGCTACAGCTCAAC		
Xuhw89/Allele specific	UHW89-BF	TCTCCAAGAGGGGAGAGACA	4-bp indel	126
	UHW89-R	TTCCTCTACCCATGAATCTAGCA		
Xucw96/CAPS	UCW96-BF	GGATGATCCCATGGATTCTACA	*Msp*I	632 + 363 + 268
	UCW96-R	TTGGTTAATCTATATGCATACTGCAAC		

CAPS, cleaved amplified polymorphic sequence; dCAPS, degenerate CAPS; LDN, *T. durum* cultivar Langdon.
[a]The bold A is a degenerate base pair (original sequence is T) that generates a unique *Nla*III restriction site in LDN.

Triticeae Repetitive Element Database (TREP) or in TIGR Cereal Repeats databases. Two had no significant similarity with any known protein or EST in any organism. The BLASTN searches using the sequences from the remaining five rice genes (*OSJNBa0026E05.13*, *OSJNBa0026E05.15*, *OSJNBa0026E05.18*, *OSJNBa0026E05.19-1* and *OSJNBa 0026E05.24*) revealed significant similarities to several *Triticeae* ESTs (Table 1). Specific primer pairs were designed that successfully amplified fragments from LDN genomic DNA for four of these ESTs (Table 1: BQ609014, BQ789353, BE444066, AL826407). The PCR products of these four ESTs were used as probes for hybridization with genomic DNA Southern blots of the two parental lines digested with multiple restriction enzymes. Hybridization results from EST BQ609014 showed multiple bands suggesting that this gene was a member of a multigene family. The other three ESTs showed one or two bands, indicating that they correspond to single copy or low copy-number genes. Despite of the large number of restriction enzymes used in the screening (24), no polymorphisms were detected between the parental lines. Therefore, PCR-markers were developed for these ESTs.

Developing PCR markers for BQ789353, BE444066 and AL826407

BQ789353 The BQ789353 primers (Table 1) amplified a 466-bp product from LDN and from nulli-tetrasomic line N6AT6B but failed to amplify any product from N6BT6A; hence, these primers were considered to be B-genome specific. This 6B locus was designated *Xuhw83*. As the 466-bp LDN and DIC sequences were identical, an additional sequence was obtained from a BAC clone that included the *Xuhw83* locus. Of the eight positive BAC clones detected with the 466-bp UHW83 probe (Table 2), only one (770E02) was assigned to the B genome.

A 5.5-kb *Hin*dIII fragment from BAC 770E02 containing *Xuhw83* was sequenced. Two new primers, UHW83-BF and UHW83-R (Table 3) were designed from a region 1-kb downstream from the BQ789353 primers (Table 1). These primers were B-genome specific, as determined by amplification of the nulli-tetrasomic lines. One single nucleotide polymorphism (SNP) was identified and used to develop a CAPS marker. Digestion of the amplification product with restriction enzyme *Hae*III produced fragments of 307-bp and 106-bp in LDN(DIC-6B) and a single 413-bp fragment in LDN (Fig. 1a). This polymorphism was used to map *Xuhw83* in the critical RSLs (Table 4) completely linked to *Xucw79* (Fig. 2).

BE444066 A PCR product of approx. 750-bp was amplified from LDN genomic DNA using the primers indicated in Table 1. Because the nulli-tetrasomic analysis showed that these primers were not genome-specific, PCR products were cloned from LDN to identify clones corresponding to the two different genomes. Digestion of the clones with restriction

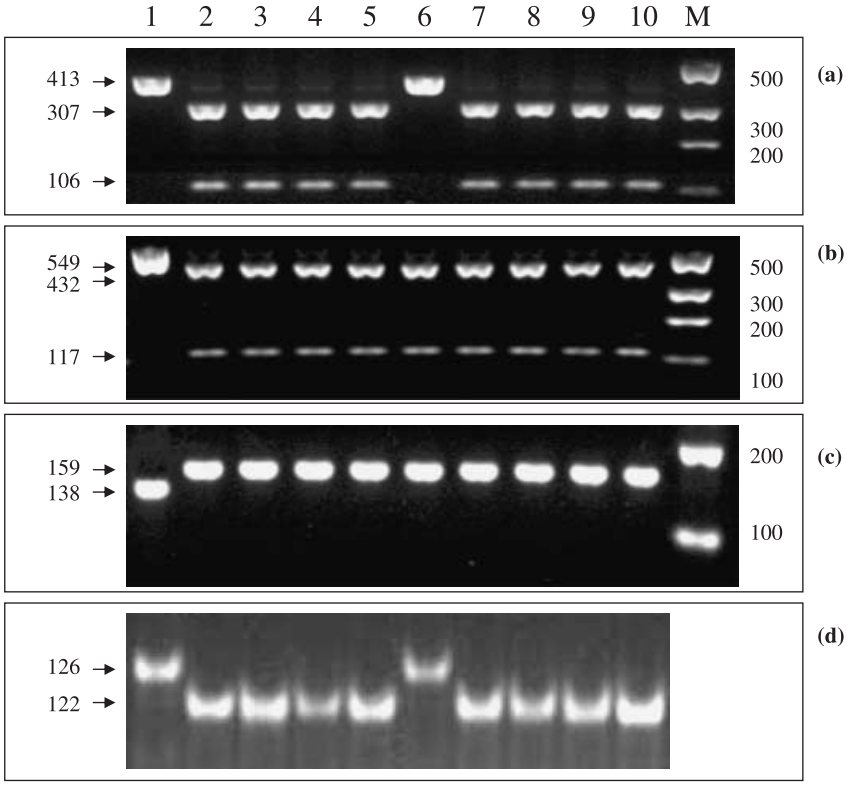

Fig. 1 (a) Cleavage amplified polymorphic sequence (CAPS) marker for locus *Xuhw83*; polymerase chain reaction (PCR) products digested with restriction enzyme *Hae*III. (b) CAPS marker for locus *Xuhw84*; PCR products digested with restriction enzyme *Sty*I. (c) Degenerate CAPS marker for locus *Xuhw86*; PCR products were digested with restriction enzyme *Nla*III. (d) Indel marker for locus *Xuhw89*. 1, LDN; 2, DIC; 3, RSL113; 4, RSL116; 5, RSL8; 6, RSL121; 7, RSL110; 8, RSL117; 9, RSL28; 10, RSL119; M, DNA ladder. The PCR and restriction products from markers *Xuhw83*, *Xuhw84* and *Xuhw86* were separated on 1.8% agarose gel; PCR products from marker *Xuhw89* were separated on 6% polyacrylamide gel. Sizes are in bp.

Table 4 Graphical genotypes of the subset of recombinant substitution lines (RSLs) previously characterized for grain protein content (GPC) (Olmos *et al*., 2003)

RSL	Xcdo 365	Xucw 75	Xucw 74	Xucw 73	Xucw 79	Xuhw 83	Xuhw 89	Xuhw 86	Gpc-B1	Xuhw 84	Xucw 71	Xucw 96	Xucw 70	Xucw 69	Xucw 67	Xucw 65
113	L	D	D	D	D	D	D	D	D	D	D	D	D	–	D	D
116	L	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
8	L	L	L	D	D	D	D	D	D	D	D	D	D	D	D	D
121	L	L	L	L	L	L	L	D	D	D	D	D	D	D	D	D
110	D	D	D	D	D	D	D	D	D	D	D	D	L	L	L	L
117	D	D	D	D	D	D	D	D	D	D	D	D	L	L	L	L
28	D	D	D	D	D	D	D	D	D	D	L	L	L	L	L	L
119	D	D	D	D	D	D	D	D	D	D	D	D	D	L	L	L

D, DIC (dicoccoides) alleles; L, LDN (cv. Langdon) alleles. A change between gray and white cells indicates a recombination event. Four additional markers were mapped between previous flanking markers *Xucw79* and *Xucw71*.

enzyme *Bam*HI identified two types of clones, and one clone per group was sequenced. Genome-specific primers were designed and tested in nulli-tetrasomic lines N6AT6B and N6BT6A. The B-genome-specific primers UHW84-BF and UHW84-R (Table 3) amplified a 549-bp fragment from a locus designated *Xuhw84*. Comparison of the LDN and DIC sequences revealed the presence of one SNP that was used to develop a CAPS marker. Digestion of the amplification product with restriction enzyme *Sty*I produced fragments of 432-bp and 117-bp in DIC and 549-bp in LDN (Fig. 1b). These polymorphisms were used to map *Xuhw84* in the critical recombinant plants (Table 4) completely linked to the targeted *Gpc-B1* locus (Fig. 2).

AL826407 Hybridization of a Southern blot, including DNAs from nulli-tetrasomic lines digested with restriction enzyme *Hin*dIII with the probe amplified by the AL826407 primers (Table 1), showed that the AL826407 corresponded to a locus designated *Xucw90* on homoeologous group 5. Therefore, this gene was excluded as a candidate for the wheat *Gpc-B1* gene.

Table 4 summarizes the graphical genotypes (Young & Tanksley, 1989) of the eight critical homozygous RSLs for which the GPC phenotype was available from previous detailed field studies (Olmos *et al*., 2003). These RSLs have recombination events close to the *Gpc-B1* gene. In Table 4, white cells with an 'L' indicate Langdon alleles, whereas gray cells with a 'D' indicate LDN(DIC-6B) alleles. The wheat

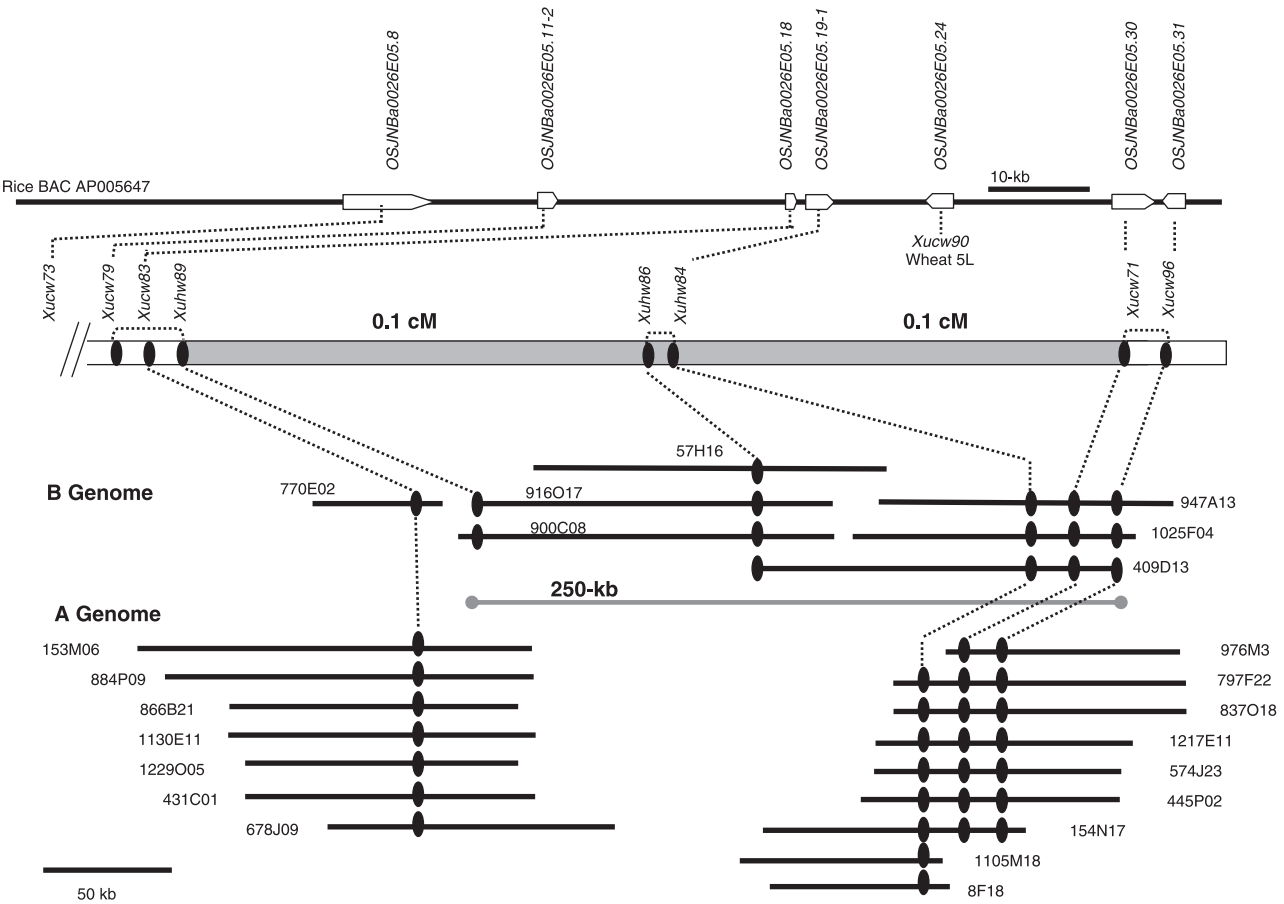

Fig. 2 Microcolinearity between the *Gpc-B1* region in wheat chromosome 6B and rice chromosome 2. The positions of the genes in the rice genomic sequence (top) are compared with the genetic and physical maps of the colinear region in wheat (bottom). The gray area represents the 0.2-cM region of the wheat genetic map including the *Gpc-B1* locus.

region between the flanking markers, *Xuhw83* and *Xucw71*, which includes the *Gpc-B1* gene, corresponds to a 36-kb region in rice BAC AP005647 (Fig. 2).

Physical map

The RSL65 BAC library was hybridized with probes for loci *Xucw71*, *Xuhw83* and *Xuhw84* (Table 3). Probes for loci *Xuhw84* and *Xucw71* detected three BACs from the B genome and nine BAC clones for the A genome, whereas distal locus *Xuhw83* hybridized with a single BAC clone from the B genome (770E02) and with seven from the A genome (Table 2).

A genome contig Among the nine BAC clones from the proximal contig, 1105M18 and 8F18 hybridized with the *Xuhw84* probe but not with *Xucw71*, facilitating the orientation of this contig relative to the genetic map (Fig. 2). No overlap was detected between the two protruding BACs from the A-genome proximal contig (1105M18 and 8F18) and the seven A-genome BACs from the distal contig selected with probe *Xucw83* (Fig. 2). As the main objective of our project was to clone the *Gpc-B1* gene from the B genome, no further efforts were made to close the gap in the physical map of the A genome.

B genome contig Distal locus *Xuhw83* provided a single small BAC clone from the B genome (770E02) with no clear overlap with the clones from the proximal contig (Fig. 2). Therefore, we focused our initial efforts for the physical mapping in the proximal B genome contig. Probes from the *Xucw71* and *Xuhw84* loci hybridized with the same 40-kb *Hin*dIII fragment. This result indicated that these two genes, located 31-kb apart in rice, were less than 40-kb apart in the B genome of wheat. The two genes hybridized with the same fragment in all three BACs; thus it was not possible to orient this contig relative to the genetic map.

To determine the orientation of the contig relative to the genetic map, we sequenced the ends of BAC clones 409D13 and 947A13 (Fig. 2). The SP6-end of BAC clone 409D13 revealed a wheat gene homologous to the colinear rice gene *OSJNBa0026E05.31* from BAC AP005647 (Fig. 2). This locus was designated *Xucw96*. Primers UCW96-BF and UCW96-R (Table 3) were confirmed to be B-genome specific

by nulli-tetrasomic analysis. Comparison of the DIC and LDN B-genome sequences revealed the presence of one SNP that was used to develop a CAPS marker. Digestion of the amplification product with restriction enzyme *Msp*I produced fragments of 632-bp, 363-bp, and 268-bp in LDN and fragments of 900-bp and 363-bp in DIC. Marker *Xucw96* was mapped completely linked to *Xucw71* and proximal to *Xuhw84* in a colinear order with rice (Fig. 2). Based on this result, it was inferred that the SP6-end of wheat BAC 409D13 was the proximal end and therefore that the T7-end should be facing the gap in the B genome contig.

The locus detected by the specific primers for the T7-end of BAC 409D13 was designated *Xuhw86*. Primers 5′-GTTT-GAGGGGATCACACACC-3′ and 5′-GCCTGTTTCCAT-ACCCATGT-3′ were used to amplify an 887-bp fragment from LDN genomic DNA and an 886-bp fragment from DIC genomic DNA. Comparison of the two B-genome sequences revealed the presence of one SNP that was used to develop a dCAPS marker (Michaels & Amasino, 1998) for the *Xuhw86* locus (Table 3). Primer UHW86-BF included a degenerate A in the second bp from the 3′ end (the original sequence was T), creating a unique *Nla*III restriction site in the amplification product from LDN. We used this primer in combination with the B-genome-specific primer UHW86-R (Table 3) to amplify a 159-bp fragment from DIC and a 158-bp fragment from LDN, which, after digestion with *Nla*III, produced a polymorphism between LDN(DIC-6B) 159-bp and LDN (138-bp and 20-bp) (Fig. 1c). This polymorphism was used to map *Xuhw86* in the critical recombinant plants completely linked to *Xuhw84* (Table 4, Fig. 2). Based on these results, we confirmed that the T7-end of BAC 409D13 (*Xuhw86*), was the end of the proximal B-genome contig facing the gap in the physical map.

The T7-end of BAC 409D13 was selected as the starting point for another step in the chromosome walking. However, the marker *Xuhw86* sequence was found unsuitable for screening the BAC library by hybridization because of its high similarity to a GAG/POL Polyprotein COPIA-like retro element. Sequencing of a downstream region, 3-kb from the T7-end of BAC 409D13, revealed a sequence with no significant similarity to any known repetitive element. Specific primers were designed to amplify a 966-bp PCR product from this region, and this second locus was designated *Xuhw86a* (Table 1).

Hybridization of the PCR product from this locus with the tetraploid BAC library revealed three positive BAC clones, 916O17, 900C08 and 57H16, in addition to BAC clone 409D13 (Table 2, Fig. 2). Analysis of the *Hin*dIII fingerprints of these clones revealed common bands with BAC clone 409D13, hence extending the B-genome contig. The ends of BAC 916O17 were sequenced and specific primers were designed for both ends. The specific primers for the SP6-end of BAC 916O17 amplified the same product from BAC 409D13 but not from BAC 947A13 or 1025F04 indicating that this was the proximal end of the BAC. The primers designed for the distal T7-end of BAC 916O17, amplified a 126-bp PCR product from LDN genomic DNA and a 122-bp PCR product from DIC genomic DNA, corresponding to a locus designated *Xuhw89* (Table 3, Fig. 1d).

This polymorphism was mapped on the critical RSLs (Table 4) and showed that *Xuhw89* was completely linked to *Xuhw83* and one crossover distal to the *Gpc-B1* gene (Fig. 2). Based on these results, we concluded that the *Gpc-B1* gene was located within the BAC contig determined by overlapping BAC clones 409D13 and 916O17 that span a region of approx. 250 kb, as estimated from the *Hin*dIII fingerprints.

High-throughput marker *Xuhw89*

The 4 bp polymorphism detected for *Xuhw89* was originated by an ACTT duplication in the LDN sequence that was absent in the DIC sequence. This size difference was sufficient to differentiate both alleles in 6% polyacrylamide gels, making this region an interesting target for the development of a high-throughput marker. Previous markers for the *Gpc-B1* gene *Xucw71* and *Xucw79*, and possibly a future marker for the gene itself, require the use of a restriction digest step after the PCR amplification, increasing cost and reducing the throughput of these markers (http://maswheat.ucdavis.edu/protocols/HGPC/index.htm).

To validate the usefulness of this marker in marker-assisted selection for tetraploid and hexaploid wheat breeding programmes, we screened a large collection of pasta and common wheat (Table 5). All the genotypes that were tested with the *Xuhw89* marker (39 tetraploid and 78 hexaploid wheat varieties) carried the ACTT duplication characteristic of the LDN allele.

Discussion

The grass family provides more than 80% of the world's edible dry matter (FAO, 2004) with wheat contributing 28% of this total (600 million tons per year). Therefore, the protein content of the wheat grain may have a significant impact on the amount of protein available for human consumption, and this was the rationale for the initial targeting of *Gpc-B1* for a positional cloning effort.

Positional cloning in the large genomes of temperate cereals is not a trivial exercise because of the huge size of the cereal genomes (13 000 Mb in tetraploid wheat and 16 000 Mb in hexaploid wheat; Arumuganathan & Earle, 1991). However, recent successful positional cloning projects have been reported in wheat and barley, demonstrating the feasibility of this approach. The positional cloning of disease-resistance genes *Mla* and *Rpg1* in barley (Wei *et al.*, 1999; Brueggeman *et al.*, 2002) was followed by map-based cloning reports in wheat for *Lr10* (Feuillet *et al.*, 2003), *Vrn1* (Yan *et al.*, 2003), *Vrn2* (Yan *et al.*, 2004), *Q* (Faris *et al.*, 2003), and *Lr21* (Huang *et al.*, 2003). In this report, we describe the identification of

Table 5 Wheat varieties tested with *Xuhw89*

Wheat	Country of origin	Variety name
Durum wheat (tetraploid)	France	Durelle, Durfort, Exeldur, Nefer, Neodur
	Italy	Adamello, Appio, Appulo, Capelli, Ciccio, Cirillo, Colosseo, Duilio, Karel, L35, Latino, Messapia, Ofanto, Russello SG7, San Carlo, Saragolla, Trinakria, Valbelice, Valforte, Valnova, Varano, Vitron, Zenit
	Israel	Inbar
	Mexico	Aconchi 89, Altar 84, Mexicali 75
	Tunisia	Inrat 69, Karim, Khiar
	USA	Colorado, Langdon, Kronos, WB881
Common wheat (hexaploid)	Australia	Summit
	Canada	Marquis
	Israel	Beit-Hashita, Mivhor, Nirit, Lachis, Galil, Gedera, Yarden, Ariel, A38
	USA	Accord, Aldente, Alpowa, Andrews, Anza, Barbee, Big Club 60, Blanca, Bonne Ville, Brooks, Cajeme 71, Calorwa, Canthatch, Centennial, Challenger, Colt, Copper, Dirkwin, Eltan, Express, Fieldwin, Garland, Gene, Hill 81, Hyak, Inia 66R, Klasic, Kmor, Lambert, Len, Mac Vicar, Madsen, Malcolm, Marshall, Meridian, Nainari 60 (CIMMYT), Newton, Nugaines, Owens, Penawawa, Phoenix, Pitic 62 (CIMMYT), Poco Red, Pomerelle, Probrand 755, Probred, Promontory, Ramona 50, Red Chief, Rohde, RSI 5, Rulo, Shasta, Siete Cerros 66 (CIMMYT), Sonora 64 (CIMMYT), Spillman, Stoa, Tadinia,Tammy, Tascosa, Treasure, Triumph 64, Turkey, Twin Serra, Vanna, Waduel 94, Yecora Rojo

two overlapping BAC clones including the flanking markers for the *Gpc-B1* gene, demonstrating once more that limited chromosome walks in wheat are feasible. However, the difficulties generated by the abundant repetitive sequences in the wheat genome complicate this task and justify an initial large investment in high-resolution genetic maps to reduce the chromosome walking efforts.

Wheat-rice microcolinearity

In a previous study, a 2.1-cM wheat region defined by markers *Xucw75* and *Xucw67* on wheat chromosome 6BS was shown to have a high level of microcolinearity with a 350 kb region in rice chromosome 2 (Distelfeld *et al.*, 2004). Except for the interruption in colinearity at the distal end of the analysed segment, the nine wheat genes included in this region were colinear with rice. In this study, three additional genes, represented by markers *Xuhw83*, *Xuhw84* and *Xucw96*, were found to be colinear between wheat and rice in the *Gpc-B1* region (Fig. 2).

The wheat *Xuhw84* gene, orthologous to the rice *OSJNBa0026E05.19-1* gene cosegregated with the *Gpc-B1* gene and, therefore, is a putative candidate gene for *Gpc-B1*. The function of the *Xuhw84* gene is unknown, but it has a conserved RNA recognition motif in the first and second exons, suggesting that it might be a regulatory factor involved in controlling the expression of other genes. The rice gene *OSJNBa0026E05.24*, adjacent to the *Xuhw84* orthologue, was mapped outside the *Gpc-B1* region in wheat (*Xucw90*, Fig. 2), representing an interruption of the colinearity between wheat and rice, close to the *Gpc-B1* gene. Therefore, this gene cannot be considered as a candidate for *Gpc-B1*, leaving *OSJNBa0026E05.19-1* (*Xuhw84*) as the only candidate gene for the *Gpc-B1* locus in the colinear region in rice. However, in order to validate this conclusion and prove that *Xuhw84* is the *Gpc-B1* gene, we need to sequence the complete colinear 250-kb region in wheat to verify that no other wheat genes are present in this region.

This study demonstrates the power of combining the rice genome sequence information (Goff *et al.*, 2002; Yu *et al.*, 2002) with the extensive *Triticeae* EST collections (Qi *et al.*, 2004) to establish high resolution genetic and physical maps in wheat. However, the observed interruptions in colinearity in the *Gpc-B1* region and similar observations in other microcolinearity studies suggest that a final sequencing of the wheat physical maps will be a frequently needed step in the positional cloning of wheat genes (Kilian *et al.*, 1997; Yan *et al.*, 2004).

Physical map of the *Gpc-B1* locus

The construction of a large-insert tetraploid wheat BAC library from RSL65 containing the DIC allele of the *Gpc-B1* gene was a necessary first step for the physical mapping of the *Gpc-B1* locus (Cenci *et al.*, 2003). This library is particularly useful for genes located on the B genome (such as *Gpc-B1*), as no diploid wheat matches the B genome from polyploid wheats perfectly (Dvorak & Zhang, 1990; Huang *et al.*, 2002). The large size of the inserts (average 130-kb) facilitated the construction of the physical map using only two BACs covering a region of approx. 250-kb, which includes markers *Xuhw89* and *Xucw71* flanking the *Gpc-B1* gene.

The average number of positive clones selected by hybridization with probes for genes *Xucw71*, *Xuhw83* and *Xuhw84* was 9.7, as expected from the estimated library coverage of 5.1 genome equivalents per genome (Cenci *et al.*, 2003). Theoretically, the number of clones from the A genome should

have been similar to that from the B genome, but a larger number of A-genome clones was recovered in this particular region (Table 2). This different representation of BACs from the *Gpc-B1* and *Gpc-A1* regions may be associated with an unusual result in the random sampling of the genome in the *Gpc-B1* region.

Relationship between genetic and physical distances in the *Gpc-B1* region

The *Gpc-B1* locus was mapped within the deletion bin 6BS-5 (fraction length 0.49–0.68), which is located proximal to the *Nor2* locus (Olmos *et al.*, 2003). Based on its proximal location within the chromosome arm, we expected a high physical to genetic distance ratio within the region. Cytogenetic studies have shown an exponential decrease in recombination with distance from the telomere (Dvorak & Chen, 1984; Lukaszewski & Curtis, 1993), with a rapid increase in the physical to genetic distance ratios closer to the centromere.

Most of the wheat positional cloning projects completed so far support this predicted increase of physical distances relative to genetic distances towards the centromeric regions. Physical to genetic distance ratios of 1.4 and 1.7 Mb cM^{-1} reported for the distal regions of chromosomes 1A and $5A^m$, respectively (Stein *et al.*, 2000; Yan *et al.*, 2004), are lower than the genome-wide estimate of 3 Mb cM^{-1} (Bennett & Smith, 1991). The *VRN-1* region located within a more proximal bin (fraction length 0.68–0.78) showed a physical to genetic distance ratio larger than the genome-wide estimate (4.1 Mb cM^{-1}, erroneously reported before as 6.1 Mb cM^{-1}; Yan *et al.* 2003). An exception to this general trend was reported for the *Q* locus located more distal than the *Vrn1* region, but more proximal than the 1A and $5A^m$ distal regions (fraction length 0.87; Faris *et al.*, 2003) that yielded an unusually low ratio of 0.33 Mb cM^{-1}.

The physical to genetic distance ratio of 1.25 Mb cM^{-1} found in the *Gpc-B1* region (250 kb, 0.2 cM) also seems to be exceptionally low considering its relatively proximal location. This ratio is similar to the values reported for the two available distal regions (Stein *et al.*, 2000; Yan *et al.*, 2004). The results from the *Gpc-B1* and *Q* regions suggest that exceptions to the general tendency of increased physical to genetic distance ratios towards the centromere will be encountered and will be difficult to predict a priori. Recent studies have shown that local patterns of recombination rate evolve very rapidly and can be different even between closely related species (Winckler *et al.*, 2005). Therefore, the chromosome location of a target gene for a positional cloning effort in wheat should be used only as a crude approximation of the physical to genetic distance ratio that will be encountered in the actual region.

Luo *et al.* (1998) suggested that recombination rates are reduced in regions adjacent to the *Nor* loci. Because the *Gpc-B1* gene is located within a small deletion bin (16% of the arm length) adjacent to the *Nor2* locus, we were concerned about a possible reduction in the recombination rates in this region, which could have complicated our positional cloning efforts. Fortunately, the reduced physical to genetic distance ratio observed in this study provides evidence that *Gpc-B1* lies far enough from *Nor2* to avoid any effect that this locus might have on suppressing recombination in this region.

Marker assisted selection (MAS)

Comparison of the distal end of the physical map between DIC and LDN (*Xuhw89*) yielded a useful polymorphic indel that was used to develop a codominant marker. This marker does not require digestion with restriction enzymes and therefore can be efficiently used in high-throughput screenings for the *Gpc-B1* gene. As this marker is tightly linked to the *Gpc-B1* gene (0.1 cM), only one in a 1000 gametes is expected to show recombination between this marker and the *Gpc-B1* gene. Based on this genetic distance, an efficient strategy would be to use only *Xuhw89* in the initial screenings for the *Gpc-B1* DIC allele. This *Xuhw89* marker showed polymorphisms between DIC and all the tetraploid and hexaploid lines tested and therefore should be useful in a wide variety of crosses in both pasta and common wheat breeding programmes. The proximal marker *Xucw71* can be used to confirm the absence of recombination in this small genetic interval in the most advanced lines of the breeding program. Markers external to *Xuhw89* and *Xucw71* can also be used to reduce the linkage drag during the introgression of the *Gpc-B1* gene.

In addition to the discovery of the *Xuhw89* high-throughput marker, the completion of the physical map for *Gpc-B1* is a significant step towards the cloning of this important agronomic gene. The final identification of *Gpc-B1* will enable the development of a perfect marker for this gene and more importantly, will shed light on the mechanisms responsible for the differences in GPC. Efforts are underway to sequence the 250-kb region and to determine whether additional wheat genes that are absent in the colinear region of rice are present in the wheat contig.

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