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Colinearity between the barley grain protein content (GPC) QTL on chromosome arm 6HS and the wheat *Gpc-B1* region

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Abstract Grain Protein Content (GPC) is an important determinant of grain quality in many crops, including barley and wheat. Recently, the map-based cloning of *Gpc-B1*, a wheat GPC quantitative trait locus (QTL), revealed a NAC transcription factor (*TtNAM-B1*) that was associated with increased grain protein, zinc, and iron content. In barley, a QTL for GPC was identified in a segregating population developed from a cross between 'Karl' (low GPC) and 'Lewis' (average GPC). This QTL was mapped near marker *hvm74* on chromosome 6H and was suggested as a potential orthologue for *Gpc-B1* on chromosome arm 6BS. In the current study, wheat genes that were previously mapped within a 0.8 cM segment spanning the *TtNAM-B1* gene were converted into barley molecular markers. These new markers, together with the barley *TtNAM-B1* orthologous gene (designated *HvNAM-1* hereafter) were mapped on a 0.7 cM interval encompassing the peak of the barley QTL for GPC on chromosome arm 6HS. Sequence comparison of *HvNAM-1* parental alleles showed two single nucleotide polymorphisms (SNPs) that result in two amino acid differences. Analysis of the allelic variation in a wild and cultivated barley collection revealed that the Karl haplotype was present only in nine out of 147 tested accessions. The colinearity between the wheat and barley GPC regions and the low frequency of the *HvNAM-1* haplotype associated with low GPC suggest that the barley NAC transcription factor is responsible for the GPC QTL on barley chromosome 6H.

Keywords Barley · Colinearity · Grain protein content · NAC transcription factor · QTL mapping · wheat

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Introduction

Grain protein content (GPC) is an important determinant of grain quality in many crops, including wheat (e.g., *Triticum turgidum* ssp. *durum* L., and *Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). High GPC (usually above 12%), is a desirable trait in breadmaking and pasta wheat varieties because of its positive effects on quality and nutritional value. Low GPC (below 11.5%) is a desirable trait for barley malt and beer production and is associated with increased

levels of malt extract and reduced problems with beer chill haze. In spite of its economic importance, genetic modification of GPC by conventional breeding has been slow because of the complex genetic system governing this trait and the high influence of the environment (Simmonds 1995).

A promising source of high GPC was detected in a survey of wild tetraploid wheat populations of *Triticum turgidum* L. ssp. *dicoccoides* (Körn.), referred to as DIC hereafter (Avivi 1978). Cantrell and Joppa (1991) developed complete sets of disomic substitution lines from each of the chromosomes of DIC accession into the tetraploid durum cultivar 'Langdon' (LDN). The substitution of the complete chromosome 6B of DIC into LDN (DIC-6B) showed the highest protein yield. Joppa et al. (1997) used DIC-6B and LDN as parents to develop a recombinant inbred line (RIL) population and mapped a QTL for GPC on the short arm of chromosome 6B. This QTL was converted later into a single Mendelian locus (designated *Gpc-B1*) using a secondary set of RILs and multiple field replications, and mapped within a 2.7 cM region between RFLP markers *Xcdo365* and *Xucw65* (Olmos et al. 2003). Microcolinearity between rice and wheat was established in this QTL region and was used to develop new markers for high-density mapping of this chromosome region. Using these markers and additional recombinant lines the *Gpc-B1* locus was mapped within a 0.3-cM segment (Distelfeld et al. 2004). This high-density genetic map facilitated the construction of a complete physical map spanning a 250 kb region encompassing the *Gpc-B1* gene (Distelfeld et al. 2006).

Uauy et al. (2006a) discovered that the *Gpc-B1* DIC allele accelerates leaf senescence and suggested that the differences in GPC were pleiotropic effects of the changes in senescence. The effect of *Gpc-B1* on senescence also explains the higher levels of soluble proteins and amino acids observed in flag leaves after anthesis in plants carrying the DIC *Gpc-B1* allele relative to those with the LDN allele (Kade et al. 2005). Chromosome 6B from DIC has been previously associated with higher grain mineral concentrations (Cakmak et al. 2004), an effect that was later determined to be associated with the 250 kb region including *Gpc-B1* (Distelfeld et al. 2007).

The map-based cloning of *Gpc-B1* (Uauy et al. 2006b) showed that this gene is a NAC transcription factor (*TtNAM-B1*) and that wild emmer wheat has a functional allele whereas modern wheat varieties carry a nonfunctional allele originated by a frame shift mutation. Both wild and cultivated accessions of tetraploid (*Tt* prefix) and hexaploid wheat (*Ta* prefix) have functional *NAM-B1* orthologues on chromosomes 6A (*TtNAM-A1* and *TaNAM-A1*) and 6D (*TaNAM-D1*), and closely related paralogues on chromosomes 2B (*TtNAM-B2* and *TaNAM-B2*) and 2D (*TaNAM-D2*). Reduction in RNA levels of these multiple wheat *NAM* homologs by RNA interference delayed senescence by more than 3 weeks and reduced grain protein, zinc, and iron content by more than 30% as compared to the non-transgenic control lines (Uauy et al. 2006b).

In barley, we identified two genes coding for proteins that were 98% identical to *TtNAM-B1* in the region including the five domains characteristic of NAC transcription factors. We designated these two genes as *HvNAM-1* and *HvNAM-2* and mapped them on chromosomes 6H and 2H, respectively (Uauy et al. 2006b). Barley chromosome arm 6HS was shown before to be associated with QTLs for GPC, nitrogen storage, and nitrogen remobilization using a population of 146 RILs, derived from a cross between high- and low-GPC barley varieties (See et al. 2002; Mickelson et al. 2003; Yang et al. 2004). A major QTL for GPC accounted for 40% of the variation in this trait was mapped in barley close to markers *abg458*, *hvm74*, and *mwg2029* and was suggested to be orthologous to the *Gpc-B1* gene located on wheat chromosome 6BS (See et al. 2002).

The objectives of the present study were: (1) to examine the relationships between *HvNAM-1* and the barley GPC QTL region on chromosome arm 6HS by comparative mapping using wheat markers from the *Gpc-B1* QTL region; (2) to explore the allelic diversity of the *HvNAM-1* gene among wild and cultivated barley accessions.

Materials and methods

Mapping population

A population of 146 recombinant inbred lines (RILs) was produced from a cross between 'Lewis' (Clho 15856) and 'Karl' (Clho 15487) (See et al. 2002).

Karl is a six-rowed malt barley that produces consistently lower GPC than other barley cultivars (Wesenberg et al. 1976) and Lewis is a two-rowed cultivar with average GPC (Hockett et al. 1985).

Polymerase chain reaction (PCR) and hybridization procedures

All PCRs were carried out in a 20 μl reaction volume under the following conditions: 1 denaturation cycle at 94°C for 4 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final extension cycle of 72°C for 7 min. Hybridizations of the BAC library high-density filters and Southern blots of *Hind*III BAC fingerprints were performed as described by Dubcovsky et al. (1994).

Phenotyping, genotyping and genetic mapping

Grain protein content (g kg^{-1}) was measured by See et al. (2002) in the growing seasons of 1997 (environment #1), 1998 (environment #2), and 1999 (environment #3 and #4) at the Post Farm, Montana, USA.

We focused only on chromosome 6H rather than the whole barley genome and used a modified genetic map for this chromosome that included newly developed barley markers converted from wheat markers tightly linked to *Gpc-B1*. Wheat molecular markers *Xucw74* and *Xuhw83* were previously mapped in wheat distal to *Gpc-B1* on chromosome arm 6BS, while markers *Xuhw84* and *Xucw71* were mapped proximal to *Gpc-B1* (Distelfeld et al. 2004, 2006; Uauy et al. 2006a). Sequences from these markers were used to develop barley molecular markers, which were designated with the same number as the wheat markers but with a three letter designator '*uhb*' (University of Haifa barley).

The genetic map of chromosome 6H developed by See et al. (2002) was based on F_5 plants and included 19 markers. The markers developed in the current research were mapped using DNA samples extracted from F_9 plants derived from the same population, and the original data for markers *acgc311*, *actt166*, *actt298*, *abg458*, *hvm74* and *mwg2029*. Other 6H markers were excluded from this analysis due to large segregation distortion and low LOD linkage values. In addition to the new barley markers developed in the present study, single sequence repeat (SSR) markers *bmag613*, *bmag496* and *bmag807* were added to the genetic map of chromosome 6H.

Multi-locus ordering and validation were done using the procedures described in Mester et al. (2003) and implemented in MultiPoint package (http://www.MultiQTL.com).

QTL analysis

Trait values of the genotypes grown under different years were analyzed using 'multiple-environment' model allowing testing various QTL-environment interaction hypotheses (Jansen et al. 1995; Korol et al. 1998). For an arbitrary genotype j, the trait measurement in the ith environment can be presented as;

$$x_{ij} = \mu_i + 0.5ga_i + e_{ij} \quad (1)$$

where μ_i is the mean trait value in the ith environment, g is either +1 or −1 for the two homozygote genotypes at a QTL, a_i is the effect of allele substitution at putative QTL on trait in environment i, and e_i is a random variable with zero mean and variance σ_i^2. When estimates of the residual variance σ_i^2 do not vary significantly among environments, QTL × E interaction analysis can be conducted by testing that $a_i \sim a$ for any i (Jansen et al. 1995). However, if the residual variance σ_i^2 for the detected QTL is not homogeneous across environments, then $a_i/\sigma_i = \text{const} = a/\sigma$ should be considered as a basis for testing the hypothesis of QTL × E interaction.

A maximum likelihood interval mapping procedure based on model "1" as implemented in MultiQTL package (http://www.multiqtl.com), was used to analyze the available data for chromosome 6H. In particular, QTL detection tests were based on permutations (>1000 runs), whereas bootstrap analysis (1000 runs) was employed for calculating the accuracy of the estimated parameters (QTL position, alleleic substitution effect, and residual variation) (Lebreton and Visscher 1998).

Results

Development of barley cleavage amplified polymorphic sequence (CAPS) markers

uhb74

RFLP Marker *Xucw74* was mapped 0.5 cM distal to the *Gpc-B1* gene in wheat (Distelfeld et al. 2004). Specific primers for the homologous barley ESTs, AL509077 and BU997795, were designed and used to amplify a 1,141-bp PCR fragment from the parental lines of the mapping population (Table 1). The sequences of these fragments (DQ682607 and DQ682608) revealed a single nucleotide polymorphism (SNP) that eliminated a *Taq*I restriction site in Lewis relative to Karl. Digestion of the amplification product with restriction enzyme *Taq*I produced fragments of 458-bp, +456-bp, +227-bp in Karl and 683-bp, +458-bp in Lewis (Fig. 1a).

uhb83

Marker *Xuhw83* was mapped 0.1 cM distal to the *Gpc-B1* gene in wheat (Distelfeld et al. 2006). Specific barley primers, designed based on the wheat EST and the homologous barley EST, BJ448685, were used to amplify an 827-bp PCR fragment (Table 1). The sequences of these fragments (DQ682609 and DQ682610) revealed one SNP that was used to develop a CAPS marker. Digestion of the amplification product with restriction enzyme *Ssp*I produced fragments of 502-bp, +325-bp in Karl and an 827-bp uncut fragment in Lewis (Fig. 1b).

uhb84

Marker *Xuhw84* was mapped 0.2 cM proximal to the *Gpc-B1* gene in wheat (Distelfeld et al. 2006; Uauy et al. 2006a). Primers, designed based on the wheat EST, were used to amplify a 205-bp PCR fragment from Lewis and Karl (Table 1). The sequences of these fragments (DQ682611 and DQ682612) revealed one SNP that disrupted a *Nla*III restriction site in Lewis. Digestion of the amplification product with restriction enzyme *Nla*III produced fragments of 137 + 68-bp in Karl and a 205-bp uncut fragment in Lewis (Fig. 1c).

uhb71

Marker *Xucw71* was mapped 0.3 cM proximal to the *Gpc-B1* gene in wheat (Distelfeld et al. 2004; Uauy et al. 2006a). Primers, designed based on the wheat EST were used to amplify a 170-bp PCR fragment from Lewis and Karl (Table 1). The sequences of these fragments (DQ682613 and DQ682614) revealed one SNP between Lewis and Karl that was used to develop a CAPS marker. Digestion of the amplification product with restriction enzyme *Mnl*I

Table 1 List of newly developed barley cleavage amplified polymorphic sequence (CAPS) PCR markers based on colinear wheat markers

Barley locus	Wheat locus	Triticeae EST	Primer sequences (5′–3′)	Restriction enzyme
uhb74	*Xucw74*	AL509077	AGGGGAATCGTTCCTTTCTG	*Taq*I
		BU997795	GGAGCATGTCAAACACACGA	
uhb83	*Xuhw83*	BQ789353	ACCTCCAAGTGCGTCAGC	*Ssp*I
		BJ448685	TGATCAACATCCACAATCAGAA	
uhb84	*Xuhw84*	BU970824	TGCTGGTCTTCAAGGTGTTG	*Nla*III
			TTCAAGTTTTGGTGGTGCTG	
uhb71	*Xucw71*	BQ753500	ACTTGTGGCAAAACTGAGCA	*Mnl*I
			CACAGTAGGAGGCAGCAACC	
uhb6	*TtNAM-B1*	DQ869678	GGGATCATCATCCATCAGAGA	*Mwo*I
			CGATGAGACGGCGTACAATA	
uhb7	*TtNAM-B1*	DQ869678	CAACCCCGTTCAACTGGCT	*HpyCH4*III
			TTCACGCCGGATATTTGGAC	

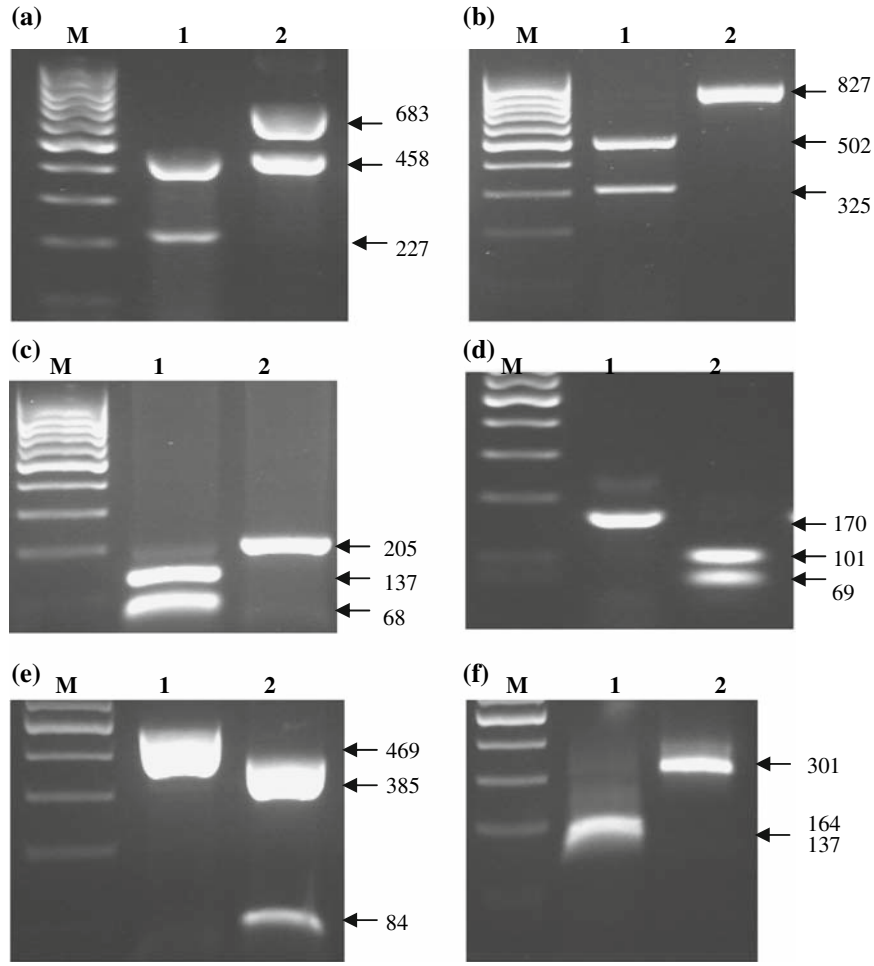

Fig. 1 Gel electrophoresis images of the six barley cleavage amplified polymorphic sequence (CAPS) markers developed in the present study. (**a**) locus *uhb74*; (**b**) *uhb83*; (**c**) *uhb84*; (**d**) *uhb71*; (**e**) *uhb6* and (**f**) *uhb7*; M = 100-bp ladder, GeneRuler™ (Fermentas), 1 = Karl, 2 = Lewis

produced a 170-bp uncut fragment in Karl and fragments of 101 + 69-bp in Lewis (Fig. 1d).

HvNAM-1—the barley orthologue of wheat *TtNAM-B1*

Analysis of the polymorphisms between the Karl and Lewis parental alleles for *HvNAM-1* (EU368851 and EU368852) revealed two SNPs that were used to develop CAPS markers, designated as *uhb6* and *uhb7*. Specific primers for *uhb6* were used to amplify a 469-bp PCR fragment from Lewis and Karl (Table 1). Digestion of the amplification product with restriction enzyme *Mwo*I produced fragments of 385 + 84-bp in Lewis and a 469-np uncut fragment in Karl (Fig. 1e). Specific primers for *uhb7* were used to amplify a 301-bp PCR fragment from Lewis and Karl (Table 1). Digestion of the amplification product with restriction enzyme *HpyCH4*III produced fragments of 164 + 137-bp in Karl and a 301-bp uncut fragment in Lewis (Fig. 1f).

Genetic map

The six new barley markers developed in the present study were mapped to a 0.7 cM region between previously mapped markers *abg458* and *hvm74* (Fig. 2). These new markers and SSR marker *bmag807* were clustered into two loci. Marker *uhb74* co-segregated with *uhb83* and *bmag807*, while marker *uhb84* co-segregated with *uhb71* and *HvNAM1* (*uhb6* and *uhb7*). Marker *uhb74* was mapped 0.7 cM proximal to marker *uhb71* whereas the orthologous wheat markers were mapped within a similar region but in

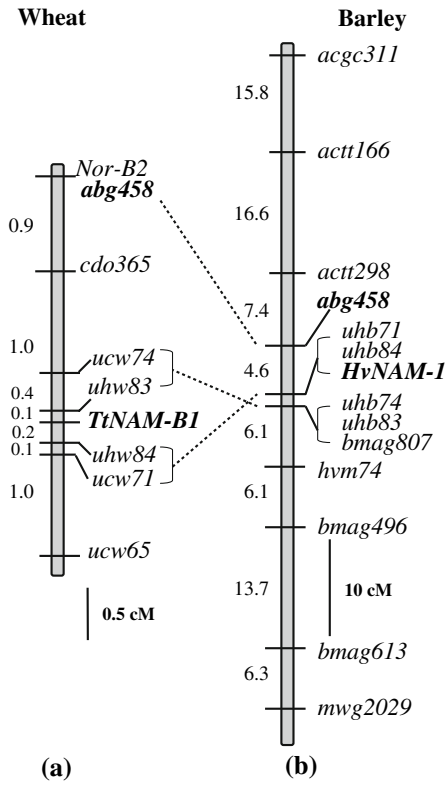

Fig. 2 Comparative mapping of the *Gpc-B1* region on chromosome arm 6BS in wheat and the colinear region on barley chromosome arm 6HS. (**a**) wheat genetic map; (**b**) barley genetic map

opposite orientation to the barley markers (Fig. 2). These results may represent a small inversion or other gene rearrangements between wheat and barley *Gpc-1* regions. However, since the development of RIL populations include multiple meiotic events, it is also possible that the critical crossover used to order the inverted loci is a result of double-crossover events that complicate the comparison. The alternative order, with the markers in the same orientation as in wheat, has a LOD value 0.3 smaller than the best marker order, indicating that it is only two times less likely.

In order to establish a better comparison between the two orthologous regions, barley RFLP marker *abg458* was converted into a wheat degenerate CAPS marker designated with the same name. Primers based on the sequence of the *abg458* probe (L43996) were used to amplify PCR products from LDN and DIC. The cloned PCR products were sequenced and one SNP between LDN and DIC was detected. Degenerate primer (TTTGTCCCCGGCAAGTAAGAT) and B-genome specific primer (ACAAACCGAACCGTGCTTT) were used to amplify 125-bp from LDN and DIC. Digestion of the amplification product with restriction enzyme *Eco*RV produced a 125-bp uncut fragment in DIC and fragments of 104 + 21-bp in LDN. Using the set of RSLs from the cross LDN × DIC-6B developed by Joppa et al. (1997) we mapped *abc458* as completely linked to the nucleolar organizing region (*Nor-B2*) on wheat chromosome 6B and 2.4 cM distal to the wheat *TtNAM-B1* gene. In barley, *abg458* was mapped 4.6 cM distal to *HvNAM-1* (*uhb6* and *uhb7*), providing an additional common marker to compare these regions (Fig. 2).

BAC library screening

Hybridizations of the barley Morex BAC library (Yu et al. 2000) were performed with probes for loci *uhb83*, *uhb84* and *HvNAM-1*. Each probe resulted in a distinct set of BACs that were confirmed by PCR (Table 2). In addition, Southern Blots of *Hind*III fingerprints for these BAC clones showed no common bands (data not shown). Interestingly, PCR with primers for *Xucw87* (proximal to *TtNAM-B1* in wheat) amplified products only from positive BAC clones for *uhb83* which is distal to *TtNAM-B1* in wheat. The presence of wheat proximal and distal *TtNAM-B1* loci within one barley BAC clone that does not include *HvNAM-1*, suggest the presence of complex rearrangements in the GPC region between the two species, or the existence of close duplicated loci.

QTL analysis

We reanalyzed the GPC data collected by See et al. (2002) using the GPC scores from different years as replicates in multiple environments. This approach together with the addition of new markers identified a major QTL for GPC on barley chromosome 6H, with a higher LOD score (60.3) than that reported by See et al. (2002) (LOD = 14). A QTL interval of 1.4 cM was established at the intersection points of the QTL peak showing a decrease of one LOD score. In the present study, the peak of the GPC QTL was mapped at the *HvNAM-1* locus and within the *abg458-hvm74* interval (Fig. 3). In all four environments tested, the

Table 2 Positive clones from barley Morex BAC library for loci *uhb83*, *uhb84*, *HvNAM-1* and *HvNAM-2*

Locus	Primers used to amplify the probe (5′–3′)	Numbers and addresses of barley BAC clones	
uhb83	AGCCACGGAGAGAACTGATG	5	120M05, 187N10, 568I17, 663F13, 795A09
(1200-bp)	TGATCAACATCCACAATCAGAA		
uhb84	TCATTTCTGCGACTGTGAGG	17	27N19, 55O11, 70M16, 74B19, 90N14, 204C15, 205C11, 224O18, 251L02, 303P12, 500D20, 555B13, 562M07, 580P13, 661F14, 763C23, 765A04
(1200-bp)	CCTGCTTTCTTCTCGTCACC		
HvNAM-1	GCTCCGACCAAACAGTTTCT	4	454A07, 433E24, 646B17, 562G13
(490-bp)	ATCCATGCAGTGGTGATGTG		
HvNAM-2	Same as for *HvNAM-1*	6	177H02, 157F07, 219G08, 577B06, 736J17, 783K16

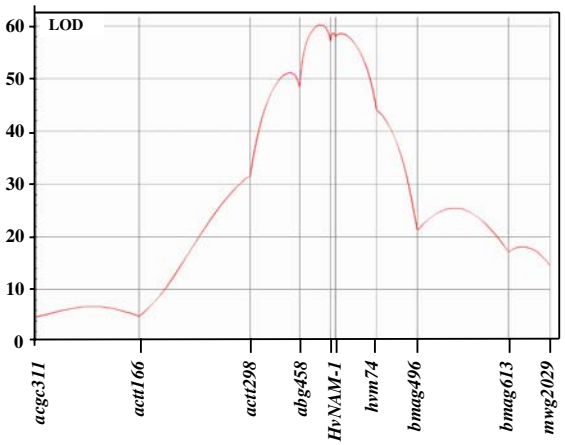

Fig. 3 QTL analysis of grain protein content (GPC) of barley chromosome 6H

Karl allele at this QTL showed a negative effect on GPC.

The bootstrap analysis presented in Table 3 indicates that there might be a difference in the percent of explained variation (PEV) by the QTL in different years. In the two environments in 1999 (#3 and #4), the *HvNAM-1* marker explained 42 and 44% of phenotypic variation (Table 3). However, the PEV results from 1997 (environment #1, 27%) and 1998 (environment #2, 51%) were more heterogeneous, indicating that the QTL effects for the three years are not similar (e.g., PEV varies from 0.27 to 0.51). Taking this heterogeneity into consideration, the estimates for the normalized QTL effects (i.e., a/σ, difference between the Karl and Lewis homozygous classes in GPC, divided by the residual standard deviation) for 1997, 1998, and 1999 were 1.2 ± 0.2, 2.0 ± 0.2, and 1.7 ± 0.1, respectively.

Allelic diversity of *HvNAM-1*

The two SNPs (*uhb6* and *uhb7*) detected between the Karl and Lewis *HvNAM-1* alleles represent substitutions in two amino acid. The first SNP, *uhb6*, is located within the third NAC domain and results in the substitution of a Proline (Karl allele) to an Alanine (Lewis allele) at position 102 of *HvNAM-1*. Comparison of the sequence of the *HvNAM-1* of Karl, Lewis and Morex with the *TtNAM* of wheat on chromosome 6A and 6B and with the NAM

Table 3 QTL × Environment analysis of GPC in barley chromosome 6H

Model	LOD	L (cM)	Environment	m	a	σ	PEV (%)
M1	60.3	43.0 ± 1.4	#1	11.6 ± 0.2	1.227 ± 0.192	1.023	26.8 ± 7.5
			#2	12.4 ± 0.2	1.400 ± 0.115	0.687	50.9 ± 5.7
			#3	13.9 ± 0.3	1.449 ± 0.148	0.852	41.9 ± 5.6
			#4	13.6 ± 0.3	1.454 ± 0.144	0.816	44.3 ± 6.0
M2	60.2	43.0 ± 1.3	#1	11.6 ± 0.2	1.228 ± 0.194	1.023	26.9 ± 7.5
			#2	12.4 ± 0.2	1.398 ± 0.120	0.686	50.8 ± 5.7
			#3	13.9 ± 0.3	1.452 ± 0.102	0.836	43.0 ± 4.1
			#4	13.6 ± 0.3	1.452 ± 0.102	0.836	43.0 ± 4.1

(a)

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Maize       GEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPI
Sorghum     GEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPI
Rice        GEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPI
wheat_6A    GEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPI
wheat_6B    GEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPI
Karl        GEHEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPI
Lewis       GEHEWYFFSPRDRKYANGARPNRAATSGYWKATGTDKPI
Morex       GEHEWYFFSPRDRKYANGARPNRAATSGYWKATGTDKPI
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(b)

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Maize       DGGGTSSS-RNNAAVGAVDS-GA-SGGTSAVSNGVGA
Sorghum     DGGGTSSSSRNNAAAGAVDS-GACSGGANAVTNGVGA
Rice        MSSSTV---DRSSA-GAVADTGDAANGANTMANGVGA
wheat_6B    MSSST----AD-MA-GAVDN-G--GGNAVNAMSTYVD
wheat_6A    MSSST----AD-MA-GAA-------GNAVNAMSAMNV
Morex_6H    MSSST----ADDMA-GAVDV-SD-GGNAVNAM--YVD
Lewis_6H    MSSST----ADDMA-GAVDV-SD-GGNAVNAM--YVD
Karl_6H     MSSST----ADDMA-GAVDV-SD-GGNTVNAM--YVD
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Fig. 4 Alignment of amino acid sequences of Karl, Lewis and Morex alleles of *HvNAM-1* as compared to the orthologous genes in wheat chromosome 6A and 6B and the homologues NAM genes in maize, sorghum and rice. (**a**) *uhb6*—the third NAC sub-domain; (**b**) *uhb7*—part of the C-terminal end. Sequences were aligned using CLUSTALW

orthologues in maize, sorghum and rice showed that the Lewis allele carries an exceptional NAC domain sequence at this position (Fig. 4a). The *uhb7* SNP is located outside the NAC domain (position 357), in the C-terminal end of *HvNAM-1*, and results in an Alanine in the Lewis allele and a Threonine in the Karl allele of *HvNAM-1*. For the *uhb7* SNP, the Lewis allele was the one identical to wheat (Fig. 4b). Based on the amino acid combinations at these two positions the Karl allele was designated 'PT' and the Lewis allele 'AA'.

Analyses of the allelic diversity for *HvNAM-1* (markers *uhb6* and *uhb7*) among wild (*Hordeum vulgare* ssp. *spontaneum*) and cultivated barley accessions are summarized in Table 4 and Appendix 1. Among the 64 *H. spontaneum* accessions analyzed in this study, the Karl PT haplotype was observed in only one accession from Israel and the Lewis 'AA' haplotype in two accessions from Libya and Iran. All the other 61 *H. spontaneum* accessions showed a combination of these two amino acids designated 'PA' (*uhb6* = P; *uhb7* = A). The high frequency of the PA haplotype together with the presence of this same amino acid combination in the wheat orthologue (Fig. 4a), suggest that 'PA' is the ancestral haplotype. The most parsimonious explanation for the haplotypes found in this study is a P to A mutation in *uhb6* generating the Lewis haplotype and an A to T mutation in *uhb7* generating the Karl allele.

Table 4 Allelic diversity for *HvNAM-1* (*uhb6* and *uhb7*) among wild barley (*Hordeum vulgare* ssp. *spontaneum*) and cultivated barley (*Hordeum vulgare* ssp. *vulgare*) accessions

Haplotype	PT (Karl)	PA	AA (Lewis)
H. spontaneum	1	61	2
H. vulgare	8	16	59

The combinations of *uhb6* and *uhb7* showed three haplotypes; 'PT'—Karl allele (*uhb6* = P; *uhb7* = T), 'PA'—presumably the ancestral allele (*uhb6* = P; *uhb7* = A) and 'AA'—Lewis allele (*uhb6* = A; *uhb7* = A)

Among the cultivated *H. vulgare* accessions the ancestral 'PA' haplotype was found in 16 accessions mainly from Japan, China and Korea (Appendix 1). Most of the *H. vulgare* accessions used in this study (59) showed the Lewis haplotype. The Karl PT haplotype was detected in only 8 cultivated accessions, including collections from Nepal, China and Ethiopia. The accession from Nepal (Everest) is one of the progenitors of Karl and Lousy (CIho 7147) and it is likely to be the donor of the unique low GPC allele.

Discussion

Colinearity between wheat and barley in the *NAM-1* region

The current study support previous results showing a high degree of conserved colinearity between chromosome arm 6HS of barley and the short arm of group 6 chromosomes of wheat. Dubcovsky et al. (1996) have shown that RFLP markers *psr167*, *abg466*, *psr8*, *mwg652* and *abg458* were colinear between wheat chromosome $6A^m$ and barley 6HS. This was further supported by Weng and Lazar (2002) that showed conservation of gene order between chromosome 6BS and 6HS and by the location of QTLs for GPC in these orthologous chromosome arms (Olmos et al. 2003; See et al. 2002).

However, our genetic map showed some putative exceptions to this colinearity. Four genes flanking *HvNAM*-1 and *TtNAM-B1* were mapped in opposite orientation between wheat and barley suggesting the presence of a small inversion or gene rearrangements (Fig. 2). These four genes were mapped to two loci in

barley separated by a single recombination event, and the likelihood of the alternative order is not very different from that of the best order. Therefore, we cannot completely rule out a similar gene order in wheat and barley, masked by the presence of multiple crossover events in this region. The multiple meiotic events that occurred during the development of the RILs increase the probability of such events.

In the current barley genetic map *abg458* is 4.6 cM distal to the GPC QTL, a position that is consistent with other barley maps (Kleinhofs et al. 1993; Kunzel et al. 2000; Li et al. 2003). In the present study, we mapped *Xucw458* in wheat linked to the *Nor-B2* locus, and 2.4 cM distal to *TtNAM-B1* (Fig. 2). The distal location of *abg458* relative to the GPC QTL in both wheat and barley indicates that the possible inversion or gene rearrangements between wheat and barley around the *NAM* gene are localized within a small chromosome segment. Regardless of this putative small rearrangement, the wheat *Gpc-B1* QTL and the barley GPC QTL are both located in colinear regions, proximal to *abg458*.

The barley GPC QTL

We have recently shown that the *TtNAM-B1* gene was responsible for the *Gpc-B1* QTL in wheat (Uauy et al. 2006b). In the present study, the orthologous barley gene, *HvNAM-1*, was mapped, together with four closely linked markers under the peak of a barley QTL for GPC on chromosome 6HS (Fig. 3). These results suggest that the wheat and barley genes are orthologous and therefore, we hypothesized that sequence polymorphism in *HvNAM-1* might provide a possible explanation for the GPC QTL on chromosome arm 6HS.

Kade et al. (2005) observed higher levels of soluble proteins and amino acids in flag leaves at anthesis in plants carrying the *TtNAM-B1* allele from wild wheat (high-GPC) relative to those with the LDN allele (low-GPC). Later in development, the higher level of protein content in the grain was associated with lower residual N content in the leaves (Uauy et al. 2006b). If *HvNAM-1* is the functional orthologue of *TtNAM-B1*, we expect to find similar differences between the two alternative *HvNAM-1* alleles. A possible evidence for such similarity could be found in the co-localization of QTLs for GPC, flag leaf nitrate and soluble organic nitrogen reported by Mickelson et al. (2003) for plants grown in 2001 (but not in 2000). These results indicate that the Lewis *HvNAM-1* allele (high-GPC) was associated with lower soluble nitrogen levels in the leaves during grain filling period (about 2 weeks after anthesis). A possible explanation of the higher N content in the grain and the lower N content in the leaves in Lewis might be a higher efficiency of nitrogen remobilization relative to Karl, similar to the case of the wheat NAM gene.

HvNAM-1 allelic diversity

Modern barley varieties appear to be relatively fixed for the high GPC, while modern durum and bread wheat varieties appear to be fixed for the low GPC. Since malting barley markets demand low GPC barley breeders have sought to introgress novel alleles into their breeding populations. The low GPC allele was introduced into barley breeding programs through the release of the variety Karl (Wesenberg et al. 1976). Karl was renowned for its unusual low and stable grain protein percentage (Burger et al. 1979), but failed as a cultivar due to poor grain yield and straw strength. One of the objectives of the present study was to test whether sequence polymorphism between Karl and Lewis can provide a possible explanation for the unusual low GPC of Karl. Our *HvNAM-1* allelic diversity survey showed that the Karl haplotype ('PT' haplotype) was rare (9 out of 147 accessions). This finding parallels the low frequency of low GPC phenotypes in barley. Analysis of Karl's pedigree also suggests a Nepalese landrace as the source for this rare low-GPC allele.

All NAC-domain proteins have a common configuration consisting of a conserved amino-terminal NAC domain region and a highly variable C-terminal region. The DNA-binding ability is associated with the NAC domain, whereas the C-terminal regions of several NAC proteins have been associated with transcriptional activation domains (reviewed by Olsen et al. 2005). Therefore, the amino acid substitution in the third NAC domain (*uhb6*) might have an effect on the DNA binding ability of *HvNAM-1* and the substitution of the non-polar Alanine into the polar amino acid Threonine might change the transcription activation of Karl *HvNAM-1*. The 'PA' and 'AA' haplotypes are

abundant among cultivated barley accessions and therefore it is tempting to speculate that the Alanine to Threonine substitution might be associated with the low GPC phenotype of Karl. Nevertheless, we cannot rule out the possibility that the unique Karl phenotype might be related to other mutations in the *HvNAM-1* regulatory regions or to a gene tightly linked to *HvNAM-1*.

Conclusions

The results presented in this study showed that the *Gpc-B1* QTL from wheat is orthologous to the chromosome 6HS GPC QTL from barley. The barley molecular markers developed in this region will be useful tools in breeding programs aimed to introgress the low-GPC allele from Karl into elite barley cultivars. The co-localization of QTLs and other recent findings suggest that the *Gpc-B1* region in wheat and the *Gpc-H1* region in barley affect nitrogen metabolism during plant senescence.

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Appendix 1 Allelic diversity for *HvNAM-1* (markers *uhb6* and *uhb7*) among wild and cultivated barley accessions

Gene bank No.	ssp.	Source	Origin/Comments	*uhb6*	*uhb7*
Lewis, CIho 15856	*vulgare*	USDA	Pedigree: Hector/Klages	A	A
karl, CIho 15487	*vulgare*	USDA	Pedigree: Traill//Good Delta/Everest/3/Traill	P	T
Hector, CIho 15514	*vulgare*	USDA	Canada	A	A
Klages, CIho 15478	*vulgare*	USDA	USA	A	A
Everest, Clho 4105	*vulgare*	USDA	Mt. Everest, Nepal	P	T
Lousy, Clho 7147	*vulgare*	USDA	Pedigree: Good Delta/Everest	P	T
2686	*spontaneum*	IPK	Iran	A	A
9819	*spontaneum*	IPK	Lybia	A	A
180046	*spontaneum*	ICARDA	Iraq	P	A
180069	*spontaneum*	ICARDA	Iraq	P	A
181595	*spontaneum*	ICARDA	Iraq	P	A
181597	*spontaneum*	ICARDA	Iraq	P	A
181598	*spontaneum*	ICARDA	Iraq	P	A
181601	*spontaneum*	ICARDA	Iraq	P	A
181602	*spontaneum*	ICARDA	Iraq	P	A
181603	*spontaneum*	ICARDA	Iraq	P	A
181604	*spontaneum*	ICARDA	Iraq	P	A
181605	*spontaneum*	ICARDA	Iraq	P	A
181606	*spontaneum*	ICARDA	Iraq	P	A
181608	*spontaneum*	ICARDA	Iraq	P	A
181609	*spontaneum*	ICARDA	Iraq	P	A
181610	*spontaneum*	ICARDA	Iraq	P	A
181655	*spontaneum*	ICARDA	Iraq	P	A
181656	*spontaneum*	ICARDA	Iraq	P	A
181250	*spontaneum*	ICARDA	Syria	P	A
181271	*spontaneum*	ICARDA	Syria	P	A
181333	*spontaneum*	ICARDA	Syria	P	A
181391	*spontaneum*	ICARDA	Jordan	P	A

Appendix 1 continued

Gene bank No.	ssp.	Source	Origin/Comments	*uhb6*	*uhb7*
181425	*spontaneum*	ICARDA	Jordan	P	A
181468	*spontaneum*	ICARDA	Syria	P	A
181547	*spontaneum*	ICARDA	Lebanon	P	A
181549	*spontaneum*	ICARDA	Syria	P	A
181568	*spontaneum*	ICARDA	Lebanon	P	A
181574	*spontaneum*	ICARDA	Lebanon	P	A
181575	*spontaneum*	ICARDA	Lebanon	P	A
296889	*spontaneum*	USDA	Israel	P	A
296901	*spontaneum*	USDA	Israel	P	A
296909	*spontaneum*	USDA	Israel	P	A
296912	*spontaneum*	USDA	Israel	P	A
296913	*spontaneum*	USDA	Israel	P	A
296919	*spontaneum*	USDA	Israel	P	A
269920	*spontaneum*	USDA	Israel	P	A
269922	*spontaneum*	USDA	Israel	P	A
296928	*spontaneum*	USDA	Israel	P	A
296932	*spontaneum*	USDA	Israel	P	A
296941	*spontaneum*	USDA	Israel	P	A
296942	*spontaneum*	USDA	Israel	P	A
296944	*spontaneum*	USDA	Israel	P	A
296945	*spontaneum*	USDA	Israel	P	A
296951	*spontaneum*	USDA	Israel	P	A
354947	*spontaneum*	USDA	Israel	P	A
366431	*spontaneum*	USDA	Afghanistan	P	A
391070	*spontaneum*	USDA	Israel	P	A
2684	*spontaneum*	IPK	Iran	P	A
2685	*spontaneum*	IPK	Iran	P	A
2688	*spontaneum*	IPK	Iran	P	A
2689	*spontaneum*	IPK	Iran	P	A
2690	*spontaneum*	IPK	Iran	P	A
2691	*spontaneum*	IPK	Iran	P	A
2692	*spontaneum*	IPK	Iran	P	A
2882	*spontaneum*	IPK	Iran	P	A
9719	*spontaneum*	IPK	Lybia	P	A
9721	*spontaneum*	IPK	Lybia	P	A
9823	*spontaneum*	IPK	Marocco	P	A
9826	*spontaneum*	IPK	Marocco	P	A
9840	*spontaneum*	IPK	Lybia	P	A
11017	*spontaneum*	IPK	Greece	P	A
11506	*spontaneum*	IPK	Greece	P	A
11509	*spontaneum*	IPK	Greece	P	A
296918	*spontaneum*	USDA	Israel	P	T
PI 467829	*vulgare*	USDA	England	A	A
PI 406263	*vulgare*	USDA	Germany	A	A
PI 290195	*vulgare*	USDA	Germany	A	A

Appendix 1 continued

Gene bank No.	ssp.	Source	Origin/Comments	*uhb6*	*uhb7*
PI 175505	*vulgare*	USDA	Finland	A	A
BR05838	*vulgare*	IPK	dist. nutans carbonera	A	A
BR05858	*vulgare*	IPK	dist. nutans martonvasari	A	A
BR05896	*vulgare*	IPK	dist.medicum anatolien	A	A
BR05948	*vulgare*	IPK	dist. nigricans mandschurei	A	A
BR05949	*vulgare*	IPK	dist. erectum hokudai no. 1	A	A
BR05969	*vulgare*	IPK	dist. nutans australische fruche	A	A
BR05983	*vulgare*	IPK	intermedium gymnanomalum	A	A
BR05995	*vulgare*	IPK	deficiens steudelii abessinien	A	A
BR010701	*vulgare*	IPK	vulg. hybernum lyallpur	A	A
BR010789	*vulgare*	IPK	vulg. wisconsin H42 (linie)	A	A
BR010708	*vulgare*	IPK	dist. nutans bannerts	A	A
BR013150	*vulgare*	IPK	fap1 2158 B BR013150	A	A
BR013156	*vulgare*	IPK	fap 1 2158 H	A	A
BR013158	*vulgare*	IPK	fap1 2158 L	A	A
BR018705	*vulgare*	IPK	ucnw c177	A	A
BR019389	*vulgare*	IPK	npc 0006	A	A
BR038255	*vulgare*	IPK	dist. glabrierectum sanalta	A	A
BR038414	*vulgare*	IPK	dist. nutans agio	A	A
C001	*vulgare*	Okayama U.	China	A	A
C004	*vulgare*	Okayama U.	China	A	A
C005	*vulgare*	Okayama U.	China	A	A
C009	*vulgare*	Okayama U.	China	A	A
C307	*vulgare*	Okayama U.	China	A	A
J232	*vulgare*	Okayama U.	Japan	A	A
J307	*vulgare*	Okayama U.	Japan	A	A
J519	*vulgare*	Okayama U.	Japan	A	A
K362	*vulgare*	Okayama U.	Korea	A	A
K366	*vulgare*	Okayama U.	Korea	A	A
K420	*vulgare*	Okayama U.	Korea	A	A
K714	*vulgare*	Okayama U.	Korea	A	A
T267	*vulgare*	Okayama U.	Turkey	A	A
T268	*vulgare*	Okayama U.	Turkey	A	A
T438	*vulgare*	Okayama U.	Turkey	A	A
T568	*vulgare*	Okayama U.	Turkey	A	A
T670	*vulgare*	Okayama U.	Turkey	A	A
T867	*vulgare*	Okayama U.	Turkey	A	A
T868	*vulgare*	Okayama U.	Turkey	A	A
U029	*vulgare*	Okayama U.	Europe	A	A
U032	*vulgare*	Okayama U.	Europe	A	A
U041	*vulgare*	Okayama U.	Europe	A	A
U045	*vulgare*	Okayama U.	Europe	A	A
U054	*vulgare*	Okayama U.	Europe	A	A
U055	*vulgare*	Okayama U.	Europe	A	A
U376	*vulgare*	Okayama U.	Russia	A	A

Appendix 1 continued

Gene bank No.	ssp.	Source	Origin/Comments	*uhb6*	*uhb7*
U629	*vulgare*	Okayama U.	Europe	A	A
U641	*vulgare*	Okayama U.	Europe	A	A
U645	*vulgare*	Okayama U.	Europe	A	A
U650	*vulgare*	Okayama U.	Europe	A	A
U659	*vulgare*	Okayama U.	Europe	A	A
A014	*vulgare*	Okayama U.	America	A	A
A031	*vulgare*	Okayama U.	America	A	A
T629	*vulgare*	Okayama U.	Turkey	A	A
PI 190755	*vulgare*	USDA	Japan	P	A
BR05815	*vulgare*	IPK	dist. nutans triumf	P	A
BR010621	*vulgare*	IPK	vulg. hybernum aegyptische	P	A
BR011929	*vulgare*	IPK	ucnwc72a	P	A
BR015670	*vulgare*	IPK	deficiens erythraeum foa II	P	A
BR026085	*vulgare*	IPK	siglah	P	A
BR038451	*vulgare*	IPK	dist. nutans pfaelzer land	P	A
BR040419	*vulgare*	IPK	hexastichon hybernum abarik	P	A
C615	*vulgare*	Okayama U.	China	P	A
K698	*vulgare*	Okayama U.	Korea	P	A
K711	*vulgare*	Okayama U.	Korea	P	A
C038	*vulgare*	Okayama U.	China	P	A
I338	*vulgare*	Okayama U.	South-West Asia including India	P	A
I437	*vulgare*	Okayama U.	South-West Asia including India	P	A
K101	*vulgare*	Okayama U.	Korea	P	A
K728	*vulgare*	Okayama U.	Korea	P	A
BR038322	*vulgare*	IPK	deficiens deficiens fehlgerste	P	T
C616	*vulgare*	Okayama U.	China	P	T
I317	*vulgare*	Okayama U.	South-West Asia including India	P	T
E469	*vulgare*	Okayama U.	Ethiopia	P	T
E878	*vulgare*	Okayama U.	Ethiopia	P	T

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