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B. Beecher · E.D. Smidansky · D. See
T.K. Blake · M.J. Giroux

Mapping and sequence analysis of barley *hordoindolines*

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Abstract Barley (*Hordeum vulgare* L.) cultivars vary in traits such as grain hardness and malt quality. However, little is known about the genetic basis of these grain quality traits in barley, while more is known about the basis of grain hardness in wheat (*Triticum aestivum* L.). Puroindolines are endosperm-specific proteins found in wheat and barley, as well as other members of the Triticeae. In wheat, variation of *puroindoline* sequence is associated with most of the variability in wheat grain texture. However, no information exists on sequence variation of the barley homologs of *puroindolines*, the *hordoindolines*. We have therefore chosen to isolate and characterize the *hordoindoline* (*hin*) sequences of eight North American barley cultivars. The barley sequences contain numerous non-conservative amino-acid substitutions relative to their wheat counterparts. However, no significant rearrangements were found in either *hinA* or *hinB* of barley. Three *hinA* and two *hinB* sequence types were found among the eight barley cultivars examined, indicating substantial allelic variation at this locus. The *hinB* sequence variability was used to map *hinB* to the short arm of chromosome 5H in a Steptoe/Morex mapping population, which is coincident with the previously mapped location of *hinA* and *Gsp* (grain-softness protein). This chromosomal location also coincides with a small barley malt-extract QTL, suggesting that *hordoindoline* sequence variation may play a small role in barley grain quality. Efforts to correlate barley seed textural differences and malting quality with *hordoindoline* sequence type are ongoing.

Keywords Barley · Wheat · *Puroindoline*s · *Hordoindoline*s · Grain hardness

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B. Beecher · E.D. Smidansky · D. See · T.K. Blake · M.J. Giroux (✉)
Department of Plant Sciences and Plant Pathology,
Agricultural BioScience Facility, Montana State University,
Bozeman, MT 59717-3150, USA
e-mail: mgiroux@montana.edu
Tel.: +1 406-994-7877, Fax: +1 406-994-7600

Introduction

Grain quality is an important consideration in cereal crop improvement. Breeders must use the available genetic variability in the development of new cultivars. Among members of the Triticeae, endosperm texture is a grain trait that varies considerably among individual cultivars. The importance of endosperm texture differences is already well-established in wheat (*Triticum aestivum* L.), where grain hardness is one of the primary determinants of end-product quality. Soft wheats have a softer endosperm texture, require less energy to mill, and yield smaller particles upon milling than do hard wheats (Symes 1965). The flours of the two types behave differently as well; soft wheats make superior cakes, while hard wheats make superior breads (reviewed in Morris and Rose 1996). Grain-hardness variation also exists among barley (*Hordeum vulgare* L.) cultivars. However, the endosperm texture of barley has received little attention. This is perhaps unfortunate, because some studies have suggested a relationship between endosperm texture and malting quality (Brennan et al. 1996; Thomas et al. 1996). In addition, it has been shown that endosperm particle size exerts a significant effect on the digestion of barley in ruminants (Bowman et al. 1996). Substantial advances in the selection for both malting and feed quality could, therefore, at least partially depend on the grain textural differences inherent in the barley grain.

The genetic basis of endosperm texture in wheat has been described (Symes 1965; Baker 1977). Only one major locus called *Hardness* (*Ha*) appears to control most of the variability of kernel hardness in this species. The *Ha* locus resides on the short arm of chromosome 5D in wheat (Mattern et al. 1973; Law et al. 1978). Three structurally related genes have been identified that are closely linked to *Ha* (Rahman et al. 1994; Sourdille et al. 1996; Giroux and Morris 1998). They are *puroindoline a* (*pinA*), *puroindoline b* (*pinB*), and *Gsp-1a*. All hard wheats characterized to-date have a sequence alteration in either *pinA* or *pinB* (Giroux and Morris 1998). The most-common mutations observed are either

a null mutation for *pinA*, or point mutations in the coding sequence of *pinB* (Giroux and Morris 1998; Lillemo and Morris 2000). Direct evidence for the involvement of *pinA* and *pinB* in rice grain texture has recently been reported. Expression of wheat *puroindolines* in transgenic rice plants conferred a softer grain endosperm texture (Krishnamurthy and Giroux, submitted).

Puroindoline-like sequences are found in other members of the Triticeae, including barley, rye and oat (Tanchak et al. 1998; Gautier et al. 2000). However, little research has centered on either the expression or allelic variation of *puroindoline* homologs in species other than wheat. This appears to be a potentially rewarding area of investigation due to the similarity of barley and wheat. As in wheat, the short arm of barley chromosome 7 (5H) contains numerous grain quality quantitative trait loci (QTLs) (Mather et al. 1997; Rouvès et al. 1996; Powell et al. 1997). This region also appears to be involved in grain texture-dependent traits such as milling energy and the level of fine-grind extract, as well as malt-extract viscosity (Thomas et al. 1996; Mather et al. 1997). Textural differences have been noted among European barleys: good-malting cultivars appear to be softer in texture than poor-malting cultivars (Brennan et al. 1996). Interestingly, the wheat *puroindolines* and the *hordoindoline a* gene reside at this chromosomal location (Rouvès et al. 1996; Sourdille et al. 1996; Giroux and Morris 1998). Because *puroindoline* sequence-differences have been implicated in wheat endosperm texture, we chose to investigate the degree of variation that exists among the *hordoindoline* sequences of barley.

We report several items that further our understanding of the *puroindolines* of barley. First, the *hinB* locus cosegregates with the the *hinA* and *Gsp* loci on chromosome 5(H) (Rouvès et al. 1996). Second, the barley *hinA* and *hinB* DNA sequences appear to be expressed at high levels in developing endosperm. Finally, substantial allelic variation was found for both *hinA* and *hinB* among the barley cultivars examined.

Materials and methods

Plant material

The barley cultivars Baronesse (PI568246), Chinook (PI591823), Harrington (license #2126), Karl (CI15487), Lewis (CI15856), Logan (PI592784), Morex (CI15773), and Steptoe (CI15229) were used in this study. Information and seed for most of these cultivars is available from the Small Grains Collection (SGC) at the National Plant Germplasm system (http://www.ars-grin.gov/npgs/). Harrington seed may be obtained from its developers (Harvey and Rossnagel 1984). The barley mapping population consisted of 150 doubled-haploid lines (DHLs) generated from a Steptoe×Morex cross (Kleinhofs et al. 1993).

Northern analysis

Northern-blot analysis was performed by standard methods as described previously (Giroux and Morris 1997). Barley plants were grown in the field at Bozeman, Montana, in the spring/summer of 1999. RNA was prepared from 20-day post-anthesis developing barley seeds by a LiCl method (McCarthy 1986). Five micrograms of RNA were separated on a formaldehyde agarose gel and blotted to a nylon membrane. Blots were hybridized to ^{32}P-labeled probes prepared by a random primer method. Following hybridization, the membranes were washed three times at low stringency (2× SSPE, 0.1% SDS) and then two times at high-stringency (0.2×SSPE, 0.1% SDS). All washes were for 15 min at 65°C. Washed membranes were exposed to Kodak Biomax MS film at –80°C using an intensifying screen. Probes were made from the coding sequence of wheat *pinA* or *pinB*, amplified as previously described (Gautier et al. 1994). The control-blot probe was prepared from a barley actin sequence, using the barley Act8 clone kindly provided by Dr. Ron Skadsen.

PCR cloning and sequence analysis

The *hinA* sequence was amplified, using *Taq* DNA polymerase (Promega), from genomic DNA using the primers PHV5′ (5′TAGGTCTGCTTGCTTTGGTAG3′) and PA3′ (5′TCACCAGTAATAGCCAATAGTG3′). The *hinB* sequence was amplified from barley genomic DNA using the primers PB5′(5′ATGAAGACCTTATTCCTCCTA3′) and PB3′ (5′TCACCAGTAATAGCCACTAGGGAA3′). The primers PA3′, PB5′, and PB3′ have been described previously (Gautier et al. 1994). The temperature regimen used consisted of a 3 min initial denaturation step at 94°C, followed by 40 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 90 s, followed by a 5-min final extension at 72°C. The amplified products were sequenced directly using an ABI 377 DNA sequencer.

PCR analysis and mapping

The PCR-based analyses were performed using genomic DNA obtained using the method of Dellaporta et al. (1983) from leaf tissue taken from 10–20 randomly selected young plants. The allelic makeup of these samples was determined by either restriction-site analysis or single nucleotide polymorphism (SNP) analysis (See et al. 2000).

The *Fnu4HI* restriction-site analysis was performed by the following method. First, a PCR reaction was employed to produce a 444-bp product using the *hinB* primers described above. The reactions were then precipitated with sodium acetate and ethanol, air-dried, and resuspended in water. This product was digested overnight with *Fnu4HI*, and the reaction products were separated on a 2% agarose gel and visualized with ethidium bromide. The resulting fragments were used to determine the *hinB* genotype, as one allele (*hinB-1*) produces 128- and 316-bp fragments, while the other (*hinB-2*) produces 128-, 155-, and 161-bp fragments.

SNP analysis was performed according to the protocol outlined in See et al. (2000). This procedure consists of two PCR reactions. The primary reaction generates the template used for the secondary reaction, which uses labeled, allele-specific primers. In this set of experiments, the first reaction used the *hinB*-specific primers described above (Gautier et al. 1994) under PCR reaction conditions consisting of 10 µM of each dNTP, 1.5 mM MgCl2, 10 mM Tris-Cl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 ng/µl of each primer, 20 ng/µl of template DNA, 0.02 units/µl of *Taq* DNA polymerase, with cycling parameters of 92°C for 2 min, 35 temperature cycles (94°C 30 s, 51°C 20 s, 72°C 20 s), followed by a 5-min final extension at 72°C. This reaction amplified the entire *hinB*-coding region. One microliter of this primary reaction was used in a 25-µl sequence-specific SNP reaction. Reaction conditions were similar to those outlined above, with the exception that the 3′ primer was replaced by a mixture of two fluorochrome-labeled primers which differed at their 3′ bases (see Fig. 2; fluorescein label for the *hinB-2* primer, HEX label for the *hinB-2* primer). The primer concentration was lower (0.1 ng/µl) than in a standard PCR reaction. The cycling parameters were 92°C for 2 min, six temperature cycles (94°C 30 s, 46°C 20 s, 72°C 20 s), followed by 5 min at 72°C. This reaction was diluted 1/5 with 1× loading dye, separated on a sequencing gel, and scanned using an ABI 377 (Perkin Elmer Applied Biosystems, Foster City, Calif., USA).

The segregation data obtained by these methods were merged with the Steptoe/Morex DHL marker dataset available at http://grain-genes.cit.cornell.edu and the linkage maps reconstructed using Mapmaker 3.0 (Lander et al. 1987). The LOD (log of difference) score and the percentage of phenotypic variance accounted for by the *hinB* locus was taken directly from the Mapmaker QTL output.

Results

Barley hordoindoline DNA sequences are expressed in developing seeds

RNA expression analysis was performed on four barley cultivars of varying market use. Two malting-type cultivars (Harrington and Morex), as well as two feed-type cultivars (Baronesse and Steptoe), were chosen for this evaluation. The Northern-blot data indicate that sequences hybridizing strongly to both *pinA* and *pinB* of wheat are present at high levels in developing barley (Fig. 1). The barley cultivars that were evaluated did not exhibit any obvious variation in transcript level for either *hinA* or *hinB* (Fig. 1).

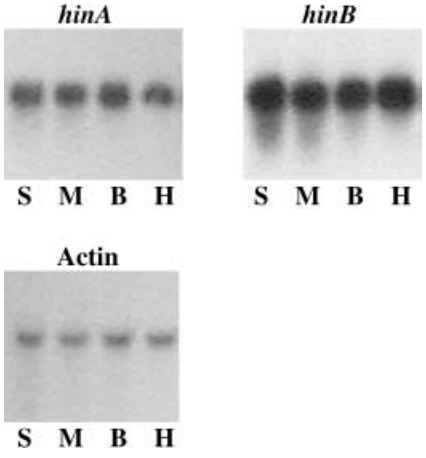

Fig. 1 Northern-blot analysis of *hinA* and *hinB* mRNA levels in developing barley seeds. Four representative cultivars were chosen: *S* Steptoe, *M* Morex, *B* Baronesse, *H* Harrington

Barley has allelic variation at both the *hinA* and *hinB* loci

To determine if barley culivars contain allelic differences in their *puroindolines*, which may relate to grain hardness, the *hinA* and *hinB* sequences from eight barley cultivars of varying endosperm texture were amplified by PCR and sequenced. The cultivars used in this study were Baronesse, Chinook, Harrington, Karl, Lewis, Logan, Morex and Steptoe. *PinA* and *pinB* homologous sequences were amplified from the genomic DNA of each of these barley cultivars. None of the cultivars contained a null mutation for either *hinA* or *hinB*. However, substantial DNA sequence variation was found for both *hinA* and *hinB* isolated from different cultivars.

At least three alleles of *hinA* are present in North American barley cultivars

HinA sequence-specific primers were used to amplify sequences from barley genomic DNA (Fig. 2). Sequence analysis of these PCR clones indicates the existence of three *hinA* alleles in barley (Fig. 2). These three sequences are designated here by the names *hinA-1*, *hinA-2*,and *hinA-3*. They show a high degree of sequence identity to

Fig. 2 Barley *hinA* DNA sequences. Three sequences homologous to *pinA* from wheat were isolated from eight barley cultivars. The *hinA*–1 allele is contained in the varieties Harrington, Chinook, Lewis and Logan. The *hinA*–2 allele was found in Morex and Baroness, while *hinA*–3 is representative of Steptoe and Karl. The primer sequence of both the sense primer and the sequence complomentary to the antisense primer are *underlined*. The 3′ antisense primer sequence has been published previously (Gautier et al. 1994)

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hinA-1    1 TAGGTCTGCTTGCTTTGGTAGCGAGCGCCGCCTTCGCGCAGTACGGAGAAGTTGTTGGCAGTTAC
hinA-2      **********************************T*****************************
hinA-3      **********************************T*****************************

hinA-1   66 GAGGGTGGTGCTGGTGGGGGTGGTGCTCAACAATGCCCCCTAGGGACAAAGCTAGATTCCTGCAG
hinA-2      ******************************A*************AA********************
hinA-3      ******************************G*************AG********************

hinA-1  131 GAATTACCTGCTAGATCGATGCACAACGATGAAGGATTTTCCGGTCACCTGGCGTTGGTGGACAT
hinA-2      ************************************************************G**
hinA-3      ************************************************************G**

hinA-1  196 GGTGGAAGGGAGGTTGTGAAGAGCTCCTTCACGATTGTTGCAGTCAGTTGAGTCAAATGCCACCG
hinA-2      ***************CTT*********************************G**************
hinA-3      ***************TTT*********************************G**************

hinA-1  261 CAATGCCGCTGCAACATCATCCAGGGATCAATCCAACGTGATCTCGGTGGTTTCTTCGGATTTCA
hinA-2      ************************************************G*******G*****
hinA-3      ************************************************G*******G*****

hinA-1  326 GCGTGATCGGACAGTCAAAGTGATACAAGCAGCCAAGAACCTGCCCCCCAGGTGCAACCAGGGCC
hinA-2      ************************************************************
hinA-3      ************************************************************

hinA-1  391 CTGCCTGCAACATCCCCAGCACTATTGGCTATTACTGGTGA
hinA-2      ***********************************
hinA-3      ***********************************
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Fig. 3 Barley *hinB* DNA sequences. Two sequences homologous to *pinB* from wheat were isolated from eight barley cultivars. The *hinB*–1 allele is contained in the varieties Chinook, Harrington, Lewis, Logan, Baroness and Morex. The *hinB*-2 allele was found in Steptoe and Karl. The sequence of the sense primer and the sequence complementary to the anti-sense primer (both previously published in Gautier et al. 1994) are *underlined* and in *normal type*. The sequences complementary to the anti-sense primers used in SNP detection are in *bold type* and are *underlined*. The sequence recognition sites for *FNu4HI* (GCNGC) are marked in *italicized bold-face type*. The 444-bp Morex *hinB* sequence was digested by *FNu4HI* into 128- and 316-bp fragments, while the 444-bp Steptoe *hinB* sequence was digested into 128-, 155-, and 161-bp fragments

one-another as well as to *pinA* of wheat (Genbank accession X69913). *HinA-1* is 97% identical to both *hinA-2* and *hinA-3*, while *hinA-2* and *hinA-3* are nearly identical (99% identity). All three barley *hinA* sequences are 90% identical with wheat *pinA*. In contrast, wheat *pinA* has only 70% and 67% DNA sequence identity with its nearest known relatives from wheat, *pinB* and *Gsp-1a*, respectively. Each barley cultivar contained a major *hinA* sequence. *HinA-1* was found in the cultivars Harrington, Chinook, Lewis and Logan, *HinA-2* in the cultivars Morex and Baronesse, and *HinA-3* in Steptoe and Karl. The high degree of sequence identity with *pinA* from wheat and other members of the Triticeae indicates that these sequences are the barley homologs of the wheat *pinA* sequences (Gautier et al. 2000). The presence of three different *hinA* sequences in the eight barley cultivars surveyed indicates that cultivated barley exhibits considerable allelic variation at the *hinA* locus. This is in contrast to hexaploid wheat, from which only one *pinA* coding sequence has thus far been identified.

At least two alleles of *hinB* are present in North American barley cultivars

Barley *hinB* sequences were isolated from the same eight barley cultivars. Like wheat *pinB*, *hinB* exhibits substantial allelic variation in barley. Sequences were amplified from the genomic DNA of eight barley cultivars using *hinB* sequence-specific primers and sequenced directly (Fig 3). Two sequences, here designated *hinB-1* and *hinB-2*, were identified among the eight barley cultivars. They exhibit a high level of identity with one another (97%) and with the *pinB* sequence of wheat (92% and 91% respectively). In contrast, wheat *pinB* has only 70% and 68% DNA sequence identity with wheat *pinA* and *Gsp-1a*, respectively. Both *hinB* sequences from barley contain an Fnu4HI restriction site at position 128, while *hinB-2* contains an additional site at position 288. *HinB-1* was found in the barley cultivars Chinook, Harrington, Lewis, Logan, Baronesse and Morex. *HinB-2* was found in the cultivars Steptoe and Karl. Each barley cultivar appeared to contain one major *hinB* sequence.

The three *hinA* alleles encode two distinct, highly identical HINA polypeptides

This study has identified three *hinA* alleles in North American barley cultivars. The predicted polypeptides encoded by *hinA-1*, *hinA-2* and *hinA-3*, as well as *pinA* from wheat, were compared (Fig. 4). The HINA-1 polypeptide sequence is identical to that reported for the barley PINA -like sequence by Gautier et al. (2000) (data not shown). *HinA-2* and *hinA-3* encode identical protein products. This sequence shares 96% identity with HINA-1. As expected, the two barley HINA sequences show a high degree of identity to PINA of wheat (85% and 86% respectively for *hinA-1* and *hinA-2* and *3*) (Gautier et al. 1994). These sequences were also compared with the 55 residues that are conserved among the PINA, PINB and GSP-1a proteins from wheat (Fig. 4). These residues were also well conserved among the HINA-1 and HINA-2/ HINA-3 sequences, with only two or three substitutions in these regions. The predicted polypeptides from both *hinA* alleles contained a threonine to alanine substitution at position 9, and a glycine to arginine substitution at position 99. The HINA-1 sequence had an additional glutamic acid to glycine substitution at position 36.

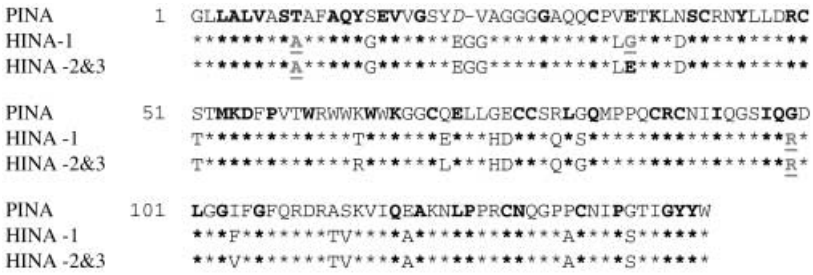


Fig. 4 Wheat PINA and barley HINA predicted peptide sequence alignment. The deduced amino-acid sequences from barley are compared to one-another as well as wheat (*pinA* Genbank accession X69913). Note that the *hinA*-2 and *hinA*-3 alleles encode identical peptides. The position of the first residue of the mature wheat *pinA* , D-22, is italicized. Residues which are conserved among the predicted peptide sequences from wheat *pinA*, *pinB*, and *GSP-1a* (Genebank accession numbers X69913, X69912 and X80381 respectively) are shown in *bold type*. The barley sequence which differs from these conserved regions is *underlined*

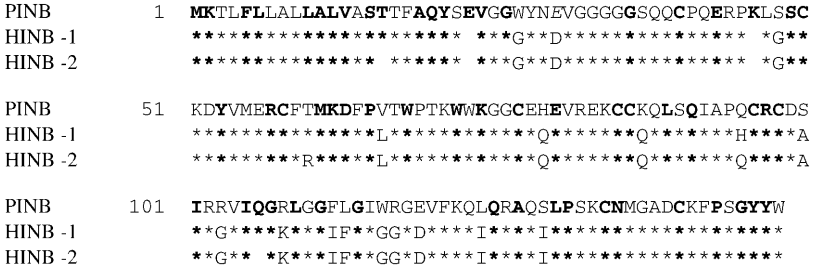
The two *hinB* alleles encode similar but distinct polypeptides

The predicted polypeptide sequences for *hinB-1* and *hinB-2*, as well as the PINB sequence from wheat, were compared to one-another (Fig. 5). These sequences are distinct from that reported for the PINB-like sequence from barley (Gautier et al. 2000, data not shown). HINB-1 and HINB-2 are 97% identical, and show 87% and 86% identity respectively with PINB from wheat. When compared to the 59 residues conserved among wheat PINA, PINB and GSP-1a sequences, both alleles have deletion of a glutamic acid residue at position 23, and a lysine to asparagine substitution at position 46 (Fig. 5). HINB-2 had two additional substitutions (a threonine to isoleucine at position 16 and a glutamine to arginine at position 106) within this highly conserved sequence. Therefore, of the four barley *hordoindoline* sequences identified here, HINB-2 shows the least conservation of the residues conserved among PINA, PINB and GSP-1a of wheat.

Fig. 5 Wheat PINB and barley HINB predicted peptide sequence alignments. The deduced amino-acid sequences from barley are compared to one-another as well as wheat (*pinB* Genbank accession X69912). The position of the first residue of the mature wheat PINB, E-30, is *italicized*. Residues which are conserved among the predicted peptide sequences of wheat *pinA*, *pinB*, and *GSP-1a* (Genebank acession numbers X69913, X69912, and X80381 respectively) are shown in *bold type*. Barley HINB residues which differ from these conserved sequences are underlined

Map position of the *hinB* locus

The allelic genotype at the *hinB* locus was determined for the Steptoe/Morex DHLs used in this mapping study (Fig. 6). Two methods were employed in this effort. The first depended upon the presence of the additional *Fnu4HI* site within *hinB-2*. *HinB* genotypes were determined by the number and size of fragments generated by *Fnu4HI*-digestion of *hinB* PCR products (Fig. 3, data not shown). This method was later replaced by the faster, more-reliable method of SNP analysis. This method uses a single nucleotide difference at position 291 to distinguish between the two alleles (Fig. 3). This permits quick, unambiguous determination of the allelic state of barley cultivars or lines (Fig. 6). These methods were used to determine the *hinB* genotype of 117 DHLs of the Steptoe×Morex mapping population. Each of the DHLs contained either *hinB-1* (from the Morex parent) or *hinB-2* (from the Steptoe parent). *HinB-1* was carried by 60 DHLs, while *hinB-2* was carried by 57 DHLs. The *hinB* locus resided on the short arm of barley chromosome 7 (Triticeae homology group 5H) near the telomers (Fig. 7a). *HinB* is inseparably linked to both *Gsp-1a* and *hinA*. These map locations are consistent with the previously characterized locations of Gsp and *pinA* in wheat (Sourdille et al. 1996; Turner et al. 1999). QTL analysis using malt-extract data (available at http://graingenes.cit.cornell.edu) co-identified this region as the site of a small malt-extract QTL (Fig. 7a). This relationship was examined further using the mean difference between DHLs carrying either of the *hinB* alleles. DHLs that carry the *hinB-1* allele average 0.48% less malt extract than *hinB-2* DHLs (Fig. 7b). This difference is small but significant, and it indicates that the *hordoindoline* allele type may explain a small portion (approximately 4.3% of the phenotypic variance) of variability in the malt extract.



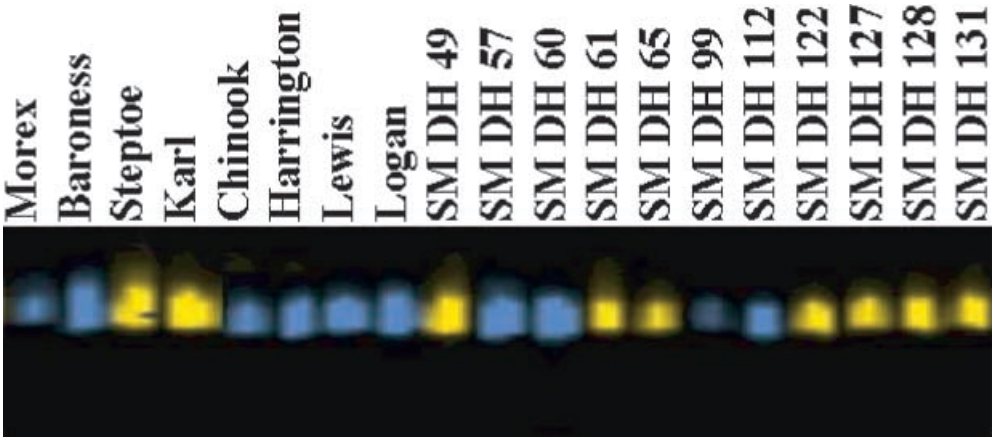
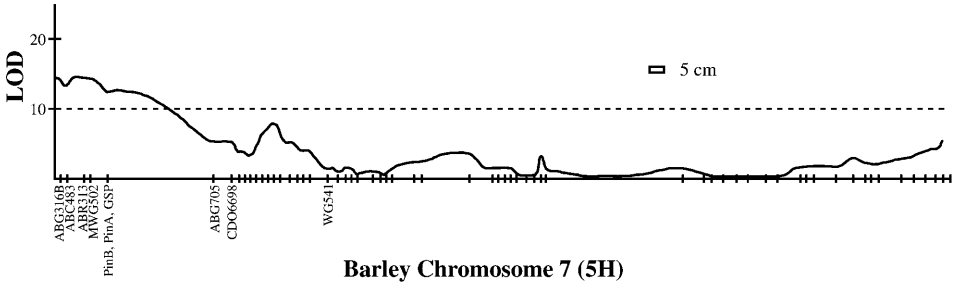


Fig. 6 Barley *hinB* allele analysis. Genotypes are easily classified according to sequence type by means of tagged, sequence-specific primers (see Fig. 2). Here, samples which carry the *hinB*–1 sequence are shown in *blue*, those carrying the *hinB*–2 allele are shown in *yellow*. To the left are the cultivars used in this study, to the right are members of the Steptoe×Morex doubled-haploid mapping population (denoted by SM DH#)

A



B

Genotype	Malt Extract (%)
Morex (*hinB-1*)	74.5
Steptoe (*hinB-2*)	71.8
hinB-1 DHLs	73.10*
hinB-2 DHLs	73.58

*Significantly different with $p<0.05$.

Fig. 7A, B A Location of the *hinB* locus in barley. The *hinB* locus maps to the short arm of chromosome 5(H) in barley. This region corresponds to a small malt-extract QTL with a LOD (log of difference) score of 1.5. Approximately 4% of the variation in malt extract may be explained by the allelic state at the *hinA*/*hinB*/*Gsp* gene complex. Malt-extract data are available at http://grain-genes.cit.cornell.edu). **B** Mean difference between DHLs carrying either of the *hinB* alleles

Barley variety characterization

Table 1 shows the cultivars grouped according to their allelic makeup at the *hinA* and *hinB* loci. Three groups of *hordoindoline* allelic types were observed. The first group is composed of the cultivars Chinook, Harrington, Lewis and Logan, which all contain *hinA-1* and *hinB-1*. The second group is composed of the cultivars Baronesse and Morex, which contain *hinA-2* and *hinB-1*. The third group comprises the cultivars Karl and Steptoe, which contain *hinA-3* and *hinB-2*.

Table 1 *Hordoindoline* sequences found in the eight barley cultivars included in this study

Cultivar	Use	*HinA*	*HinB*
Chinook	Malt	*HinA*-1	*HinB*-1
Harrington	Malt	*HinA*-1	*HinB*-1
Lewis	Feed	*HinA*-1	*HinB*-1
Logan	Feed	*HinA*-1	*HinB*-1
Baroness	Feed	*HinA*-2	*HinB*-1
Morex	Malt	*HinA*-2	*HinB*-1
Karl	Malt	*HinA*-3	*HinB*-2
Steptoe	Feed	*HinA*-3	*HinB*-2

Discussion

Barley cultivars vary in traits such as grain hardness and malt quality. These traits, endosperm texture (milling energy) and malting quality, may be associated with one another (Brennan et al. 1996). The *puroindoline* genes have been implicated in endosperm texture differences in barley's close relative, wheat (Sourdille et al. 1996; Giroux and Morris 1998). In addition the wheat *puroindoline* genes have recently been shown to affect the endosperm texture of transformed rice when expressed as transgenes (Krishnamurthy and Giroux, submitted). Our goal in this study was to better-characterize the *hordoindoline* genes of barley with the view that this information could be used in future barley improvement efforts. We have, therefore, examined the mRNA levels, sequence, and chromosomal locations of the *hordoindoline* genes in barley.

The effect of *puroindoline* genes upon grain softness is presumed to be dependent upon their expression in developing seeds. In fact, one of the two major mutations associated with grain hardness in wheat is a null for *pinA* that does not accumulate a *pinA* transcript (Giroux and Morris 1998). Northern-blot analysis indicated that developing barley seeds expressed sequences homologous to both *pinA* and *pinB* of wheat (Fig. 1). The level of mRNA was high and appears to be similar among the tested barley cultivars. It would therefore appear that *hinA* and *hinB* are expressed at reasonably high levels in the proper location (developing seeds) to influence grain texture in barley. Moreover, the results indicate that hardness variation among barley cultivars is not likely to be due differences in the level of *hordoindolines* mRNA levels.

Barley cultivars differ in traits related to grain texture. In wheat it has been shown that grain textural differences are associated with allelic differences in the *puroindoline* genes (Giroux and Morris 1998). In fact, little *puroindoline* allelic variation exists among tested wheat varieties. Each single amino-acid change found in hard hexaploid wheat varieties has been interpreted as a mutation (Giroux and Morris 1998; Lillemo and Morris 2000). Any hypothesis relating the *hordoindoline* genes to grain quality traits in barley would rely on the existence of allelic variation at one or both loci. Analysis of the *hordoindoline* sequences isolated from eight barley cultivars of varying hardness indicates that substantial allelic variation exists for these genes in cultivated barley. Three *hinA* and two *hinB* alleles were identified (Figs. 2 and 3). The sequences showed substantial identity among themselves and to their homologs in wheat. We are suggesting the designations *hinA-1*, *hinA-2* and *hinA-3* for the three *hinA* allelic sequences of barley shown here (Fig. 2). Likewise we suggest that the two barley *hinB* alleles be designated as *hinB-1* and *hinB-2* (Fig. 3). Chinook, Lewis, Logan and Harrington are all 2-row barley varieties of Northern European extraction, and all carry the *#1* alleles for both *hinA* and *hinB*. Steptoe and Karl are 6-rowed varieties with parentage deriving from North Africa, and carry *hinA-3* and *hinB-2*. Morex is a 6-rowed variety of Asiatic background (the base variety is 'Manchuria'), while Baronesse is a 2-rowed drought-tolerant variety derived from a commercial breeding effort that employed relatively exotic parents. These last two varieties carry the *hinA-2* and *hinB-1* alleles. This degree of *hordoindoline* sequence diversity is perhaps surprising when compared to wheat. Little *puroindoline* sequence variation has been reported in the literature, and most of the sequence variation that exists appears to consist of single-nucleotide substitutions (Giroux and Morris 1998; Lillemo and Morris 2000). In contrast, this study of only eight barley varieties has revealed five *hordoindoline* sequences, most of which contain several nucleotide substitutions.

The *hinB* gene of barley has been mapped to the short arm of chromosome 5H, and is closely linked with the barley *hinA* gene (Rouvès et al. 1996). This region has already been identified as being partly responsible for differences in milling energy, extract viscosity, and the level of fine-grind extract (Thomas et al. 1996; Mather et al. 1997). This chromosomal location also coincides with a small barley malt-extract QTL (Fig. 7). It seems plausible that some or all of these traits could be controlled in part by the *hordoindolines*. However, variation in hardness among barley cultivars is much smaller than that among soft- and hard-wheat cultivars. It should be noted that the barley *hordoindoline* sequences contain many differences from the *puroindolines* of hexaploid wheat. These changes might impact on the degree to which they function in grain texture. In addition, measurement of malting quality is difficult because of the large role played by the environment. For instance the small malt-extract QTL mentioned in this study was not apparent in the study by Rouvès et al. 1996, although the same mapping population was used. More work will be required to resolve these issues.

Selection for improved or maintained malting quality is a challenging plant-breeding objective, because trait measurement is difficult and expensive, and because environmental variation confounds heritable variation. It was therefore reasonable to assume that this area could have been overlooked thus far by breeders. However, if in fact the *hordoindoline* alleles are found to differ in their effects on endosperm hardness, this variation could account for a portion of the grain quality trait differences that are associated with this chromosomal region. This idea can be tested now that the sequences involved have been characterized, by using mapping populations containing differences in *hordoindoline* allelic makeup and grain quality. Barley breeding efforts could benefit if this is the case, because of the relative ease by which lines can be screened by SNP analysis.

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