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ORIGINAL PAPER

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Cloning and molecular characterization of B-hordeins from *Hordeum chilense* (Roem. et Schult.)

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Abstract One of the main limitations of cereal breeding is the lack of genetic variability within cultivated crops. *Hordeum chilense* is a wild relative of *Hordeum vulgare*, which has been successfully used in the synthesis of amphiploids by crossing with *Triticum* spp. Among the agronomic traits of these new amphiploids, the allelic variation in the endosperm storage proteins and their influence on breadmaking and malting quality are of special interest. B-hordeins are sulfur rich prolamins, which account for 70–80% of the total hordein fraction in barley. In this work, rapid amplification of cDNA ends by PCR (RACE-PCR) has been used for the cloning of the full-length open reading frame (ORF) of six sequences of B3-hordeins from two lines of *H. chilense*. Two consensus sequences of 813 and 822 bp for the H1 and H7 lines, respectively, were determined by alignment of all the sequences generated. Between both lines, differences involving single base changes, which could correspond to single nucleotide polymorphisms (SNP), insertions and deletions were observed. Of these differences, only six out of the 13 within the ORF caused a change of amino acid. Two insertions/deletions of 9 and 12 bp were also observed between both lines. The derived amino acid sequences showed a similar structure to the B-hordeins from cultivated barley and other prolamins. The repetitive region is based on the repetition of the motif PQQPFPQQ. The copy number of the B3-hordeins was estimated as a minimum of nine and five copies for the H1 and H7 lines, respectively. The expression profile of the B-hordeins through the developing endosperm is also described in this work. This study of the storage proteins of *H. chilense* is a useful contribution to the knowledge of the genetic diversity available in wild relatives of cultivated barley. In addition, the origin of the different prolamins can be better understood with an in-depth knowledge of its wild equivalent.

Keywords B-hordeins · Cereal breeding · *Hordeum chilense* · Quantitative real time-PCR

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Introduction

The main protein source for many human and animal populations is cereal endosperm. In the past, improving the quality of cereal has been the focus of plant breeders and nowadays is the target of new technologies such as genetic engineering. One of the main objectives of cereal breeding is to expand the genetic variability within the different cultivated species. Wild species, related to the cultivated plants, are an important source of variability. With this intention, the amphiploid tritordeum (× *Tritordeum* Ascherson et Graebner) was obtained by crossing *Triticum turgidum* ssp. *durum* (Desf.) Husn. with *Hordeum chilense* (Roem. et Schult.) (Martin and Sanchez-Monge 1982). This cross-breeding produced additional variability for important agronomic traits. Among these, the seed storage proteins contributed by *H. chilense*, whose effect on breadmaking and malting quality have been extensively studied (Alvarez et al. 1992, 1999; Martín et al. 1999; Alvarez et al. 2001), are divergent.

The major storage proteins in barley endosperm are the prolamins, on account of their high levels of proline and glutamine. Although cultivated barley possesses reasonable variation in its storage proteins, searching for new diversity within these proteins is of interest for different breeding programs. In barley, these proteins are called hordeins and are important determining malting quality (Marchylo et al. 1986; Howard et al. 1996). Hordeins are classified into three groups: S-rich

(B and γ-hordeins), S-poor (C-hordeins) and high molecular weight (HMW), the D-hordeins, which are homologous to the HMW glutenin subunits of wheat (Shewry et al. 1995). The B-hordeins and C-hordeins account for 70–80% and 10–12% respectively of the total hordein fraction, while the D and γ groups are quantitatively minor components (Molina-Cano et al. 2001). Moreover, it was found that the B fraction had some effect on malting quality by influencing the conversion of starch into sugar and it has been suggested that lower grain nitrogen content and lower ratio of D:B hordein results in improved malting quality (Peltonen et al. 1994). In spite of much investigation of the B-hordeins, information on the sequence and structure of the B-hordeins is sparse, with only seven complete sequences having been deposited in GenBank (five B1-hordeins and two B3-hordeins).

In this work, we report the isolation of six new cDNA clones and the deduced amino acid sequences encoding the B-hordeins of *H. chilense*. A comparison with the B-hordeins from cultivated barley and the equivalent low molecular weight (LMW) glutenin subunits from wheat is also reported. This information is presented to provide an improved understanding of the structure and function of the B-hordeins for further application in breeding programs.

Materials and methods

Plant material

The diploid accession lines of *H. chilense* (Roem. et Schult.) H1, H7, H11, H16, H47, H210, H220, H252, H293, and H297 from the germplasm collection of the Instituto de Agricultura Sostenible, CSIC, Cordoba (Spain) were used in this study. Plants were grown in a greenhouse with supplementary lights providing a day/night regime of 12/12 h.

Amplification of short fragments of the B-hordein genes

Primers HoB*1–HoB*2 designed to amplify short fragments of *H. chilense* B-hordein genes are described in Table 1. These primers were designed on the basis of the published sequence of the mRNA sequence of the B3-hordein from *H. vulgare* (GenBank accession number X03103). All primers used in this study were synthesized by Sigma-Genosys (Pampisford, UK). The recovered sequences have been deposited in GenBank under accession numbers AY700787–AY700795.

DNA isolation and PCR conditions

Genomic DNA from the above *H. chilense* lines was isolated using a CTAB method (Stacey and Isaac 1994). The PCR conditions used to amplify short fragments of *H. chilense* B3-hordein gene were: 1× Gold buffer (Applied Biosystems, Foster City, Calif.), 1.5 mM $MgCl_2$, 400 μM dNTPs (Applied Biosystems), 0.5 μM of each primer, 1.25 U Amplitaq Gold (Applied Biosystems) and 100 ng of genomic DNA. The PCR amplification was run as follows: an initial step of 94°C for 10 min, and then 35 cycles of 94°C for 30 s and 70°C for 1 min, followed by 5 min at 72°C. The resulting products were gel-purified, cloned into the pGEMT-Easy vector (Promega, Madison, Wis.), and introduced into competent *Escherichia coli* (DH5α) cells by transformation. The plasmid was isolated and purified using a QIAprep Spin Miniprep Kit from Qiagen (Valencia, Calif.) and used as a sequencing template.

RNA isolation

RNA was isolated using the TRizol reagent from Gibco BRL Life Technologies (Grand Island, N.Y.) according to the manufacturer's instructions, and treated with-DNase I (RNase free) from Roche Diagnostics (Basel, Switzerland) to eliminate DNA contamination. One control PCR amplification containing RNA was routinely performed to ensure that the RNA was free of DNA.

Amplification of cDNA ends

Total RNA from the endosperm of *H. chilense* line H7 was extracted 10 days after flowering to synthesize anchored cDNA at the 5′ and 3′ ends as described by the SMART RACE cDNA Amplification Kit from Clontech (Palo Alto, Calif.). The specific primers HoB*5 and HoB*6 (Table 1) were designed using the short fragments previously sequenced to generate overlapping 5′-RACE and 3′-RACE products. The 5′ and 3′ end products were size-fractionated by gel electrophoresis, and the bands eluted using a Gel Extraction Kit from Qiagen, and cloned and sequenced as describe above. These sequences are accessible at GenBank under accession numbers AY700796–AY700800.

Full-length mRNA isolation

The sequences obtained from the 5′ and 3′ end products were used to design the primers HoB*11 and HoB*18 (Table 1) which amplify the full open reading frame (ORF) from the mRNA consensus sequence. cDNA was generated using total RNA from the *H. chilense* lines H1 and H7 by using the Superscript II Reverse Transcriptase from Invitrogen (Carlsbad, Calif.) using oligo $(dT)_{12\text{-}18}$ primers (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. These full-length gene sequences were then ligated into the pGEMT-Easy vector (Promega) and sequenced. The sequences were deposited in GenBank with accession numbers AY700801–AY700806.

Table 1 PCR primers used for cloning and characterization of B3-hordein genes from *H. chilense*

Primer	Description	Sequence (5′ → 3′)
HoB*1	External forward primer for B3-hordein	TCATCCATCTATTTTGCAACAGCTAAACCCATGC
HoB*2	External reverse primer for B3-hordein	GGGATTTGGGGTAGTTGCTGGCAACATTG
HoB*5	Specific primer for 3′ RACE	AAGGTATTCCTCCAACAGCAGTGCAGCC
HoB*6	Specific primer for 5′ RACE	TTGTTGCAACACATGACAACTGCTCTGCTG
HoB*9	Forward primer for B3-hordein quantification	CAGCAACAGTGTTGCCAACAACTACCCCAAA
HoB*10	Reverse primer for B3-hordein quantification	TGGGGTTGTTGCTGCTGAGGTTGGAC
HoB*11	Forward primer for "edge-to-edge"	AAGCACAAGTATCAAAACCAAGCAAC
HoB*13	Forward primer for genome walking	AATGCCACAACGTATTGCTAGGTC
HoB*14	Reverse primer for genome walking	TGTTGTTCTTGGAGGATGATGGAGT
HoB*18	Reverse primer for "edge-to-edge"	CCACACAAAGCCGATATGAGCA
HcITS*3	Forward primer for the ITS1 ribosomal gene	CAGACCGCGCTCGTGTCATCCAA
HcITS*4	Reverse primer for the ITS1 ribosomal gene	TGACCCCGAGTTAGGCACAGTGTTCCTTG

Quantitative real time PCR

Developing grains were harvested 4, 8, 12, and 18 days after flowering, which correspond to growth stage numbers 69, 71, 75, and 83, respectively, according to the Zadoks code (Zadoks et al. 1974). Total RNA was extracted and cDNA synthesized as described above. For real time cDNA PCR, the SYBR green dye marker was used on the ABI Prism 7000 (Applied Biosystems) together with the gene-specific primers HoB*9 and HoB*10 (Table 1). Levels of mRNA were quantified using the internal transcribed spacer (ITS1) of the 18S-5.8S-26S ribosomal genes from *H. chilense* (GenBank accession number AJ288116) using the primers HcITS*3 and HcITS*4 (Table 1). All reactions were replicated three times. Gene expression data were processed and standardized according to Muller et al. (2002).

Preparation of labelled probes

A full-length clone of the B3-hordein from *H. chilense* line H7 in pGEMT-Easy was used as the template for the generation of digoxigenin (DIG) labelled riboprobes. The riboprobe containing digoxigenin-11-2′-deoxy-uridine-5′-triphosphate (DIG-11-dUTP) from Roche Diagnostics was synthesized by PCR using the primers HoB*11 and HoB*18 (Table 1) according to the manufacturer's protocol.

Genomic southern blot analysis

Total genomic DNA was extracted from leaf tissue of the *H. chilense* lines H1 and H7 as described above. Three batches of genomic DNA (10 μg each) were digested separately with *Bam*HI, *Hin*dIII, and *Pst*I, electrophoresed through 0.8% agarose gels and blotted onto a Hybond-N^+ membrane (Amersham Biosciences) and baked for 2 h at 80°C. Hybridization and signal detection was performed according to the Roche Diagnostic protocols. Briefly, the membrane was prehybridized for 1 h with DIG Easy Hyb Granules (Roche Diagnostics) at 48°C. The DIG labelled riboprobe was then added and hybridized overnight at 48°C. After hybridization the membrane was washed twice with a solution of 0.1% (wt/vol) SDS in 2×SSC for 15 min at 65°C. The DIG Luminescent Detection Kit (Roche Diagnostics) was used for signal detection. Finally, the membrane was exposed to AGFA medical X-ray film (Agfa-Gevaert, Mortsel, Belgium) film for 1–6 h.

Bioinformatic analyses

BLASTn and BLASTp sequence identity searches were performed at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) via GeneQuest 5.05 from DNAStar (Lasergene, Madison, Wis.). Oligo 6.82 from Molecular Biology Insights (Cascade, Colo.) was used for all the primer designs. The chromatograms from the sequencing of the different fragments were edited and assembled using Sequencher 3.1.1 from Gene Codes (Ann Arbor, Mich.). Amino acid sequences were aligned using the CLUSTALX (Version 1.81) (Thompson et al. 1997). ProML in Felstein's PHYLIP package (Felsenstein 1999) was used to generate a maximum likelihood tree based on the Jones-Taylor-Thornton algorithm (Jones et al. 1992) using the previous amino acid alignment.

Results and discussion

Isolation of full-length sequences of the B-3 hordein genes

Several combinations of primers were initially designed on the basis of the B-hordein sequences from *H. vulgare*. However, only one fragment of 162 bp was amplified using the primers HoB*1 and HoB*2 (Table 1). This fragment was confirmed as B-hordein by a BLASTn search, and was used for designing the primers HoB*5 and HoB*6 (Table 1). This enabled the amplification of the 5′ and 3′ ends from cDNA synthesized from line H7 by RACE-PCR. Assembling the overlapping 5′ and 3′ ends provided the consensus sequence for the complete mRNA of a B-hordein from *H. chilense*. Subsequently,

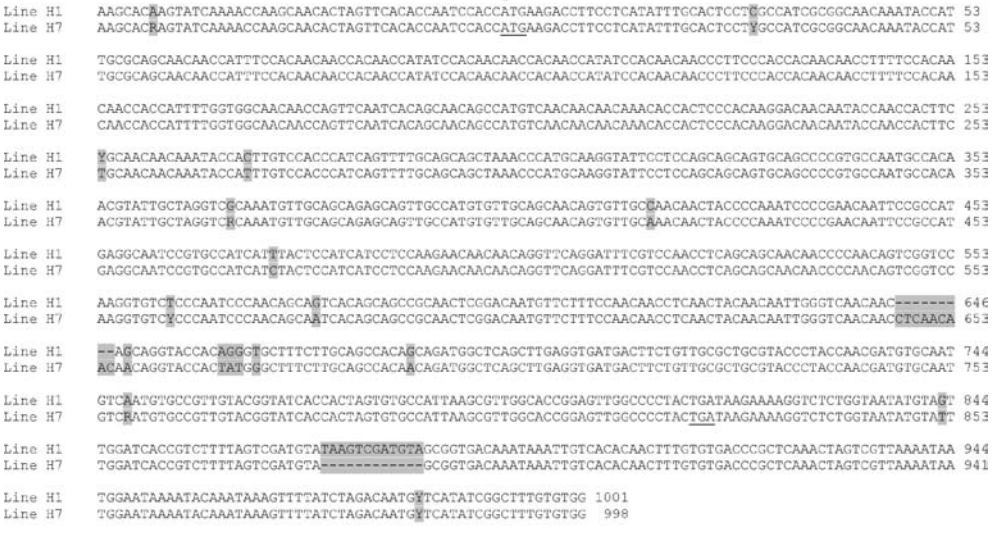
new primers were designed on the basis of the above consensus sequence to amplify the full ORFs from cDNA generated from lines H1 and H7. Enzymes with proofreading activity were used for the amplification and three different clones were sequenced in both the forward and reverse directions to minimize the possibility of PCR errors. After sequencing, three different sequences of B-hordeins for line H1 and three for line H7 were identified and the corresponding consensus sequences for both H1 and H7 lines were aligned. These are shown in Fig. 1A.

Nucleotide sequence analysis

The ORFs for H1 and H7 contained 813 and 822 bp, respectively. Differences involving single base changes, insertions and deletions were observed between both consensus sequences (Fig. 1A). Single base discrepancies were present at positions −41, 30, 254, 271, 369, 418, 474, 554, 579, 648, 659, 663, 681, 748, 843, and 982; an insertion is present at position 646–647 and a deletion between positions 871–882, all with respect to line H1 (Fig. 1B). The differences between H1 and H7 involving single bases may be used for SNP analysis and selection. Of these differences, only six of the 13 that were within the ORF caused an amino acid change. In addition, the insertion at position 646–647 did not result in the breakage of the ORF, as it is inserted as three complete codons. Considering only the clones containing the complete ORF, the SNP frequency was about one every 500 bp in the B3-hordeins of *H. chilense*. Zhang et al. (2003) have previously observed a frequency of SNPs of one per kilobase in the γ-gliadins of wheat, a frequency half that observed here for the B3-hordeins from *H. chilense*.

The ATG starting codon was not preceded by the GCCACC consensus sequence typical of most eukaryotic genes (Kozak 2001), indicating that the first methionine residue may not be included in the final protein. The 5′-untranslated region (leader sequence) contained 47 bases and lacked the AAAGA sequence implicated in the secondary structure of the mRNA found in some eukaryotic mRNAs. Likewise, no stop codon was present immediately preceding the coding sequence (CDS) but the closest was found at position –19 upstream from the ATG start codon. Conversely, the 3′-untranslated region showed a double stop codon, characteristic of the LMW and HMW glutenin subunits of wheat and the B-hordeins of *H. vulgare* but not of the gliadins of wheat (Cassidy et al. 1998). Most of prolamins sequenced to date have at least two copies of the sequence that conforms to the consensus for the polyadenylation signal sequence (AATAAA) in their 3′-untranslated regions (Dean et al. 1986). The first motif was located 60–80 nucleotides 3′ from the termination codon, whereas the second was about 50 nucleotides further downstream. Only in the family of LMW

A



B

LINE	Polymorphism position									
	-41	30	254	271	369	418	474	554	579	646-647
H1	A	C	C/T	C	G	C	T	T	G	----------
H7	A/G	C/T	T	T	A/G	A	C	C/T	A	CTCAACAAC
	648	659	663	681	748	843	871-882			982
H1	G	AGG	T	G	A	G	TAAGTCGATGTA			C/T
H7	A	TAT	G	A	A/G	T	----------			C/T

Fig. 1 **A** Alignment of consensus sequences of B3-hordeins from *H. chilense* lines H1 (*upper*) and H7 (*lower*). Discrepancies within and between both consensus sequences are *shaded* (*R* purine, *Y* pyrimidine). **B** Discrepancies between the consensus sequences of H1 and H7 indicating the putative SNPs, insertions and deletions. All the positions are indicated on the basis of the H1 consensus sequence

glutenin subunits and the B-hordein sequences from wheat and barley, respectively, there is a third motif very close to the second polyadenylation signal (four or five nucleotides further downstream) (Ciaffi et al. 1999). The B3-hordein from *H. chilense* described in this work not only presented this third polyadenylation signal but also a fourth putative polyadenylation signal (AATAAT) 42 nucleotides downstream from the first polyadenylation signal and two nucleotides upstream of the third signal (Fig. 1A). The presence of several polyadenylation signals could give rise to alternative polyadenylation sites, which may be implicated in some form of posttranscriptional regulation by modifying the transport of the mRNA to the cytoplasm and its stability and/or the initiation of translation.

Amino acid sequence analysis

The deduced amino acid sequence was determined from both consensus sequences, and BLASTp searches confirmed the similarity between the B3 and B1-hordeins from *H. vulgare* and with other prolamins. From this data both amino acid sequences from *H. chilense* were determined to be B3-hordeins. These sequences were aligned with the complete sequences of all known B-hordeins from *H. vulgare* and the most similar sequences of LMW glutenin subunits from other cereals (Fig. 2).

The translated precursor proteins of the B3-hordeins from *H. chilense* contain 271 and 274 amino acid for lines H1 and H7, respectively. These B3-hordeins present characteristics similar to those of the S-rich prolamins, especially the B-hordeins from barley and the LMW glutenin subunits from wheat (Shewry et al. 1984, 1995; Forde et al. 1985; Kreis et al. 1985; D'Ovidio et al. 1997; Cassidy et al. 1998; Ciaffi et al. 1999; Ikeda et al. 2002). The basic arrangement is a core repetitive region flanked by two non-repetitive regions, and eight cysteine residues that form intermolecular and intramolecular disulfide linkages (Shewry et al. 1984). The sequence starts with a signal peptide composed of 19 residues (1–57 bp from the start codon) at the N-terminal end of the encoded protein (MKTFLIFALLAIAATNTIA) (Fig. 2). This signal peptide is rich in hydrophobic amino acids (13 out of 19 residues) and is similar to that previously described for the B1-hordein from *H. vulgare* (Forde et al. 1985). As expected, the signal peptides are flanked by hydrophilic residues at their N-terminal (T) and C-terminal (QQQ) sites, including one basic residue near the N-terminal. There is also a three residue uncharged polar stretch (TNT) inside the hydrophobic core, similar to that of the B1-hordein of barley (Forde et al. 1985).

In many prolamins like the LMW glutenin subunits from *T. aestivum*, the signal peptide is followed by a short domain (I) that, in some cases, contains the first cysteine residue (Fig. 2). However, this short domain (I) is absent in the B3-hordeins from *H. chilense* and in all B-hordeins from *H. vulgare* as well as in the LMW glutenin subunits

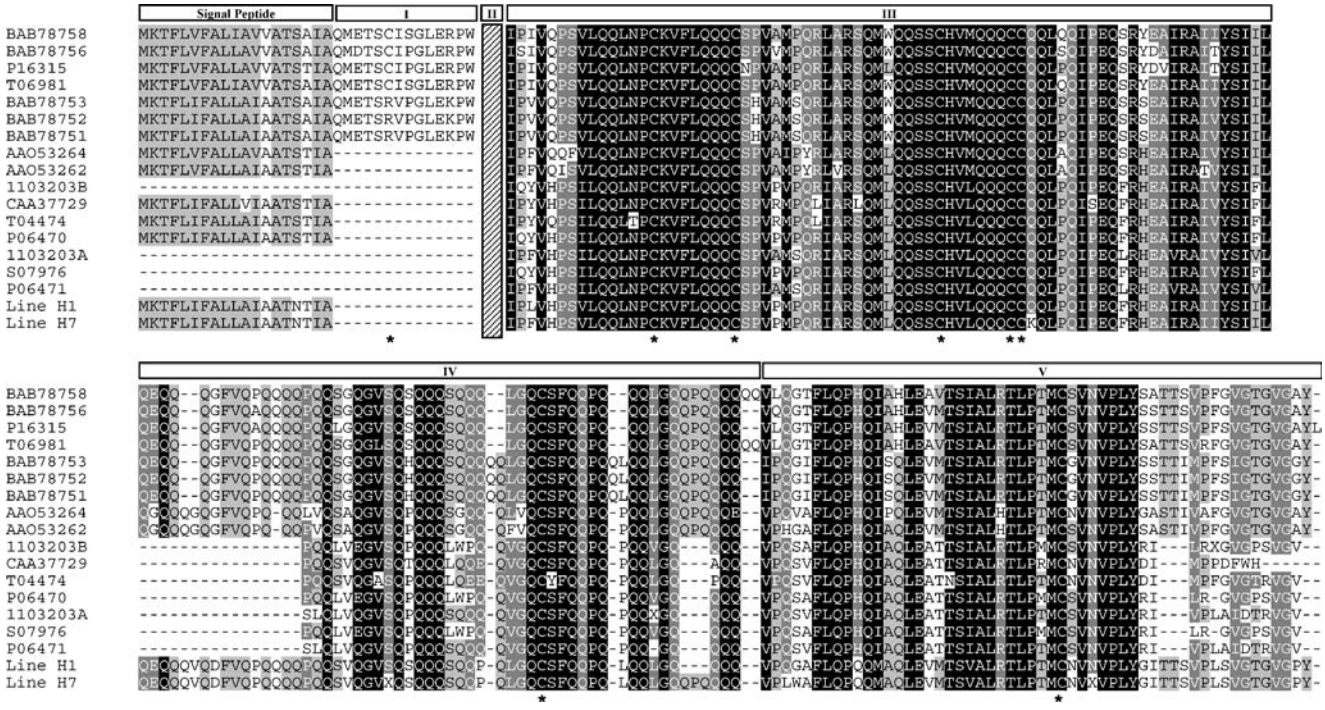


Fig. 2 Alignment of the B3-hordein sequences from the *H. chilense* lines H1 and H7 with the B1-hordeins (1103203B, S07976, P06470, T04474, and CAA37729) and the B3-hordeins (P06471 and 1103203A) from *H. vulgare*, the LMW from *T. intermedium* (AA053262 and AA053264) and the LMW type III (BAB78751, BAB78752, and BAB78753) and type IV (BAB78756, BAB78758, P16315, and T06981) sequences from *T. aestivum*. The polypeptide regions are as for the LMW in Cassidy et al. (1998). The vertical bar indicates the positions of the repetitive region (removed for this figure). Asterisks indicate the positions of the eight cysteine residues. The degree of sequence conservation is indicated on the grey scale

from *T. intermedium* (AAO53262 and AAO53264) (Fig. 2). Therefore, in the B3-hordeins described here, the signal peptide is followed directly by the repetitive domain (II), which contains 69 residues (see below) presenting the first cysteine residue near to their C-terminal end of this repetitive region. This cysteine residue is well conserved in all sequences which lack domain I (the B-hordeins and LMW subunits of *T. intermedium*).

As described for other prolamins (Cassidy et al. 1998), the repetitive domain is followed by a conserved region, which is divided into three domains (domains III, IV, and V) (Fig. 2). Domain III contains five cysteine residues, which are present in all B-hordeins from *H. chilense* and *H. vulgare* and in all LMW glutenin subunits. As shown in Fig. 2, domain III is the best conserved among all sequences in the alignment. Domain IV is characterized by a high glutamine content; the sequences from lines H1 and H7 contain 30 and 32 glutamines respectively out of their total 57 residues (Fig. 2). Tandem repeats of glutamine residues are highly variable (Anderson and Greene, 1997) and in the alignment in Fig. 2 are present mainly in short runs of 2–4 residues, suggesting a DNA slippage-mismatching of the glutamine codon during evolutionary replication (Cassidy et al. 1998). The B-hordeins from *H. vulgare* are 13 residues shorter in the N-terminal end of domain IV than the B3-hordeins from *H. chilense* and the LMW glutenin subunits from *T. aestivum* and *T. intermedium* (Fig. 2). This fact could indicate that deletion/insertion events may have occurred in these sequences. In addition, the presence of the tripeptide QPQ in the C-terminal region of domain IV, which is absent in all of B-hordeins except in line H7, could indicate another deletion/insertion event (Fig. 2). Domain IV is rich in single repeats of glutamine residues. As mentioned above, simple sequence DNA often undergoes slippage-mispairing during DNA replication. Such repeats have also been identified as hotspots of recombination (Wahls et al. 1990) that can result in unequal crossing over, leading to expansion or contraction of the original sequence. Domain IV contains a single cysteine residue only and together with its flanking sequences is highly conserved in all aligned sequences (Fig. 2). Some regions of the amino acid sequence of domain IV in the *H. chilense* lines are more similar to those of the LMW glutenin subunits of *T. aestivum* and *T. intermedium* than to the B-hordeins of *H. vulgare* (Fig. 2).

The C-terminal end of domain V presents the last cysteine residue (Fig. 2). This domain is well conserved among all sequences except in its C-terminal region, where the B3-hordeins from *H. chilense* are more similar to the LMW glutenin subunits than to the B-hordeins from *H. vulgare*.

Structure of the repeats in domain II

This domain is characterized by a series of degenerate tandem repeats (Kreis et al. 1985). The nucleotide and deduced amino acid sequences of the repeat region of the B3-hordein from *H. chilense* are shown in Fig. 3 to emphasize the motif structure. The DNA consensus sequence, based on the most frequently occurring nucleotide for each position in the codon is CCA CAA CAA CCA TTT CCA CAA CAA, and its corresponding amino acid motif is PQQPFPQQ. Variations from this consensus sequence within this repetitive region were usually due to single pair changes and the insertion or deletion of glutamine codons (Fig. 3). The repetitive domain of B3-hordein is significantly smaller than those present in the rest of the B-hordeins of cultivated barley and the LMW, except for the LMW from *T. intermedium* (accession number AA053264). In wheat, variation of the repetitive regions is well documented in γ-gliadin and other prolamins. Our observations could indicate that the process of shrinking and expansion of the repetitive region may also occur in the barley genome. Variation in the length of the repetitive region of the B-hordein and γ3-hordein genes between cultivated and wild genotypes of barley have been reported by (Kanazin et al. 1993; Piston et al. 2004).

The tandem repeat of the B3-hordeins from *H. chilense* is the same as those reported for the B1-hordeins (Forde et al. 1985), γ-secalin and C-hordein (Tatham and Shewry 1995). It is also very similar to that proposed for γ-gliadin $PFPQ_{1\text{-}2}PQQ$ (Anderson et al. 2001). As for domain IV, there is a high degree of conservation in the C-terminal end of the repetitive region between all the B-hordeins and LMW glutenin subunits of *T. intermedium* (data not shown). In the case of the LMW of *T. aestivum* this region is less conserved in comparison with those shown above, and it is divided by repetitive motifs and runs of glutamines. The fact that the con-

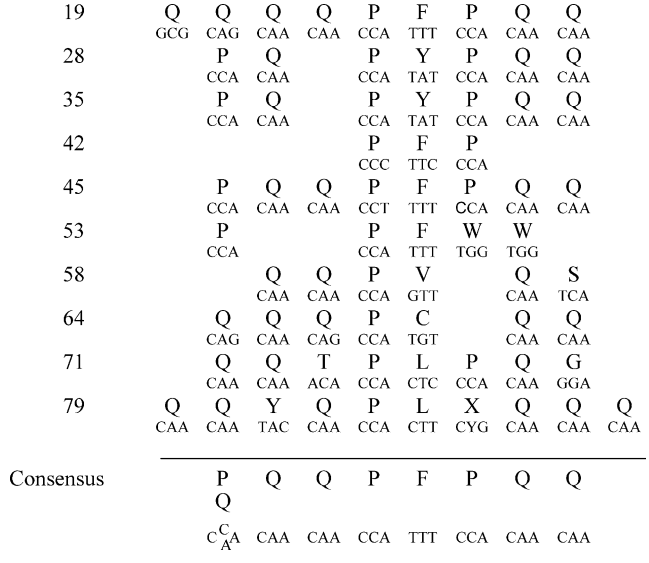


Fig. 3 Alignment of the repetitive motif for the B3-hordein from *H. chilense*. This alignment is derived from the consensus sequences from lines H1 and H7. The consensus codon repeats and the derived amino acid residue patterns (given below) are based on the most frequently occurring nucleotide for each position. Numbers on the left indicate the amino acid position in the protein sequence

served region is fragmented in the LMW of *T. aestivum* could be a consequence of events that occurred during the evolution and development of the repetitive region. In addition, the presence of this conserved region in the B-hordeins and LMW glutenin subunits of *T. intermedium* could provide evidence that it is part of the sequence that gave rise to the repetitive region.

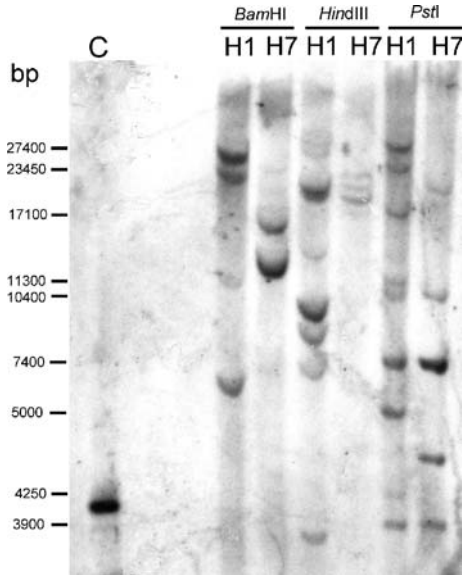


Fig. 4 Southern analysis of the B3-hordein genes from lines H1 and H7. Genomic DNA was cut with three different restriction enzymes. Lane C is a control where the B3-hordein gene from line H7 were ligated into pGEMT-Easy and linearized with *Eco*RV

Copy numbers of the B3-hordeins in *H. chilense*

The analysis of the nucleotide sequence of the B3-hordeins of both the H1 and H7 lines showed several discrepancies within and between lines, which could be explained by the fact that several copies of the gene are present at the same locus, as reported for other barley cultivars (Kanazin et al. 1993). Genomic Southern analysis was carried out in order to determine and explain the copy number of the B3-hordein genes in lines H1 and H7 of *H. chilense*. In this analysis a maximum of nine and five fragments, for H1 and H7 respectively, appeared in the genomic DNA cut with *Pst*I (Fig. 4), which would correspond to a minimum of nine copies for H1 and five for H7. Sabelli and Shewry (1991) have reported that by restriction fragment analysis of the LMW, γ and omega gliadins, one fragment corresponds to several copies. This may also be the case in the B3-hordeins from *H. chilense* and, therefore the number of copies may be greater than the number of fragments that appear. It is possible that the number of copies in *H. chilense* is similar to the 15–30 copies of the B-hordein genes at the *Hor2* locus of *H. vulgare* (Kanazin et al. 1993). The difference in the number of fragments obtained between the lines H1 and H7, whatever the restriction enzyme used, indicates variation between the lines.

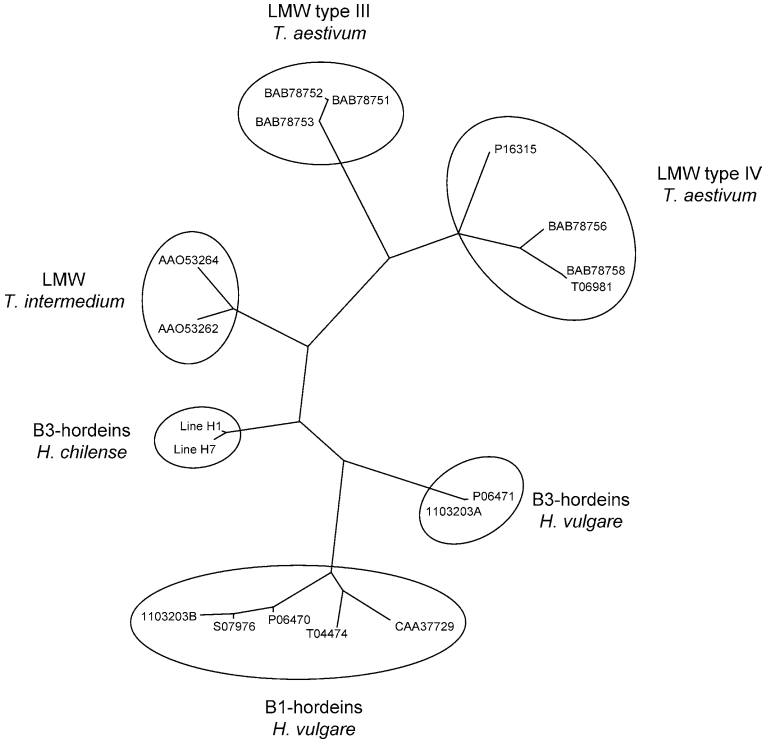


Fig. 5 Maximum likelihood tree of sequences aligned in Fig.2 based on the entire amino acid sequences. The amino acid sequences were compared by Clustal analysis with minor modifications. In the tree, lines H1 and H7 are analysed as the consensus sequence from each line. The rest of the sequences are indicated by the accession number

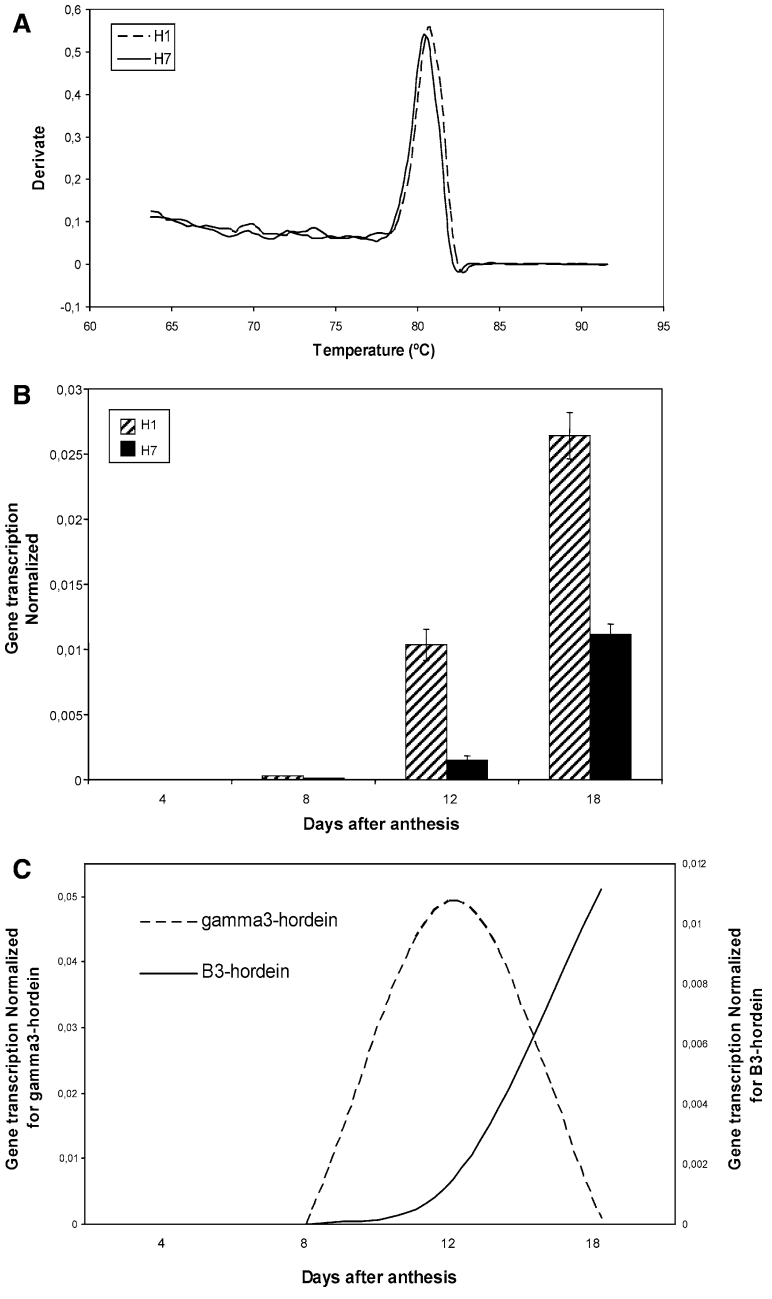


Fig. 6 Analysis by quantitative real time PCR of transcription of the B3-hordein genes from the H1 and H7 lines in developing endosperm. **A** Dissociation curves to determine the melting temperature of both H1 and H7 B3-hordein genes using HoB*9 and HoB*10 primers. **B** The fold changes in gene transcription was determined at different grain developmental stages, normalized according to Muller et al. (2002), and represented in arbitrary units. **C** Comparison of the expression levels between the γ3-hordein and the B3-hordein genes from *H. chilense*, both from line H7 at different endosperm development stages. The data of expression of γ3-hordein have been taken from Piston et al. (2004)

Interrelation of the B3-hordeins from *H. chilense*

The alignment from Fig. 2 was used to generate a parsimonious phylogenetic tree based on the Jones-Taylor-Thornton method (Thompson et al. 1997) with the options of global rearrangements and multiple jumbles (Fig. 5). In this tree, a close relationship was observed between the B3-hordeins from *H. chilense* and the B-hordeins from *H. vulgare*, especially the B3-hordeins. It is noteworthy that the homology of the LMW glutenin subunits of *T. intermedium* with both B3-hordeins of *H. chilense* is higher than the homology with other proteins from its own family, such as the LMW glutenin subunits of *T. aestivum*. In fact, the first accessions that appeared in the BLASTp results are the LMW subunits from *T. intermedium*.

Quantitative real time PCR

The storage proteins are specifically expressed throughout the development of the endosperm (Cameron-Mills and Brandt 1988; Davies et al. 1993; Piston et al. 2004). The transcription level of the B3-hordein genes was monitored by quantitative real time PCR (QRT-PCR), which is a highly sensitive technique to detect different amount of mRNA. The internal transcribed spacer (ITS1) of the 18S-5.8S-26S ribosomal genes from *H.*

chilense was used as a reference gene. ITS1 has been successfully used as reference gene in previous works (Benítez-Burraco et al. 2003; Piston et al. 2004). Dissociation curves for both the H1 and H7 B3-hordein genes are indicated in Fig. 6A. The difference in melting temperature between both sequences can be explained on the basis of one SNP at position 474 (T → C, Fig. 1B) inside the region amplified by the HoB*9 and HoB*10 primers. In the endosperm of both lines, transcription was found 8 days after flowering, but at a very low level. This result agreed with those reported by Davies et al (1993) who found that mRNA for B-hordeins in cultivated barley could be detected by in situ hybridization 7 days after anthesis. In *H. chilense* a progressive increase in the expression levels between 12 and 18 days after anthesis, which corresponds to the maximum levels of expression for both lines (Fig. 6B), was observed. Expression of the B3-hordeins was also compared with the expression of γ3-hordein of *H. chilense* (Piston et al. 2004). As shown in Fig. 6C, the pattern of expression of both storage proteins is different; for γ3-hordein the maximum level of expression occurs 12 days after anthesis and rapidly decreases to minimum level at 18 days after anthesis. Therefore, the expression of B3-hordein is delayed with respect to γ3-hordein (Fig. 6C). The implications of this pattern may be in the coordinated transport and deposition of hordeins in the developing endosperm. However, information about the processing and function of the B-hordeins is scanty and requires further elucidation.

Conclusions

The B-hordeins storage protein family has been studied in this work in two lines of *H. chilense*, a wild relative of cultivated barley. The study of wild species related to crops is of interest because they can provide valuable genetic variation for future agronomic applications and breeding programs. Differences in the sequences between lines H1 and H7, and within the sequences from the same line (SNPs, insertions and deletions) have been found. Both lines also differed in the copy number, which was estimated as a minimum of nine and five copies for H1 and H7, respectively.

The amino acid composition of the B-hordeins from *H. chilense* is similar to that of the B-hordeins of *H. vulgare*, and both are very similar to the LMW of *T. intermedium* and *T. aestivum*. Although the B3-hordeins from *H. chilense* show a more global relationship with the B-hordeins from cultivated barley, some regions of domain IV of *H. chilense* show a closer link to the LMW glutenin subunits of *T. aestivum* and *T. intermedium*, which may be due to the common origin of the LMW glutenin subunits and the B-hordeins. In wheat, the LMW glutenin subunits make a notable contribution to the bread-making quality. The high homology of the B-hordeins with the LMW glutenin subunits suggests similar bread-making influences for these B-hordeins. Moreover, the bread-making qualities obtained with different tritordeum lines suggests that the B-hordeins may make a positive contribution to bread-making quality and could be of use not only in cultivated barley or tritordeum but also in modifying wheat bread-making by interspecific transformation.

The spatial and temporal expression of these proteins, as determined by QRT-PCR, provides an improved understanding of the order of accumulation of this important protein family. In addition to this, the expression profiles presented here are useful for transformation, since promoters driving the expression of the B-hordeins can be used to specifically express genes of interest in the endosperm.

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