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ORIGINAL PAPER

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Cloning and characterization of a gamma-3 hordein mRNA (cDNA) from *Hordeum chilense* (Roem. et Schult.)

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Abstract *Hordeum chilense* is a wild relative of *H. vulgare*, cultivated barley, that has been successfully used in the synthesis of amphiploids by crossing with *Triticum* spp. These amphiploids—named generically × *Tritordeum*—have been tested under field conditions, and one of them, the hexaploid tritordeum obtained following chromosome doubling of the hybrid *H. chilense* × *T. turgidum*, shows traits of interest inherited from the barley parent. Of great interest is the allelic variation observed in the endosperm storage proteins and their influence on the breadmaking and malting quality of tritordeum. We report here two mRNA (cDNA) sequences for a gamma-3 hordein from two accession lines of *H. chilense*, H1 and H7, and their characterization by quantitative real time (QRT)-PCR in the developing endosperm. Sequences were obtained by rapid amplification of cDNA ends and "edge-to-edge" amplification of open reading frames from cDNA of *H. chilense*. Eight putative single nucleotide polymorphisms and one codon insertion were identified in the sequences of the H1 and H7 gamma-3 hordeins. The deduced amino acid sequences showed similar features to that of the gamma-3 hordein and gamma-gliadins from barley and wheat, respectively. While the repetitive motif (PQQQPF) is similar to that of the gamma-3 hordein from *H. vulgare*, there are 19 motif repeats in *H. vulgare*, whereas *H. chilense* shows 15 tandem repeats. The transcription of the genes encoding for the gamma-3 hordein were monitored by QRT-PCR: in both lines maximum transcription occurred 12 days after flowering.

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Introduction

One of the limiting factors in current breeding programs is the low genetic variability of cultivated plants. Consequently, wild species related to crop plants are important sources of genetic variability in the breeding programs. One approach to exploiting this wild genetic variation is the synthesis of amphiploids by chromosome doubling of the hybrid. Following just such a strategy, the amphiploid tritordeum (×*Tritordeum* Ascherson et Graebner) was obtained by crossing *Triticum turgidum* ssp. *durum* (Desf.) Husn. with *Hordeum chilense* (Roem. et Schult. (Martín and Sánchez-Monge 1982). *Hordeum chilense* is a wild relative of *Hordeum vulgare* (cultivated barley), and more than 250 primary tritordeum lines have been synthesized using 103 accessions of this species (Martín et al. 1999). As a consequence, the genetic variability generated for important agronomic traits is enormous, most of which derives from *H. chilense*. Among these traits, allelic variation in the endosperm storage proteins of *H. chilense* (Alvarez et al. 1999, 2001) is of particular interest in relation to their influence on the breadmaking or malting quality of tritordeum (Alvarez et al. 1992, 1994).

Prolamins are the major storage proteins of cereals. In barley, storage proteins are among the determinants of malting quality, with a negative correlation between malt extract yield and protein content (Molina-Cano et al. 2000). The prolamins of barley comprise D hordeins, which are homologous to the high-molecular-weight (HMW) glutenin subunits of wheat, sulphur-poor (S-poor) C hordeins and sulphur-rich (S-rich) B and γ hordein (Kreis and Shewry 1989). In barley, the S-rich prolamins are, quantitatively speaking, the major prolamin group, accounting for about 80–90% of the total prolamin fraction (Shewry et al. 1995). They include polymeric and monomeric components—the B and the γ hordeins, respectively. In wheat, the homoeologous gamma-gliadins are well studied, with more than 20 different sequences being registered at GenBank (Anderson et al. 2001). However, little information is available about the

structure, properties and functions of the gamma-hordeins, with only two sequences registered in GenBank—the gamma-1 and gamma-3 hordeins (accession numbers M36378 and X72628, respectively).

We report here the sequences of two mRNAs (cDNA) encoding gamma-3 hordeins from *H. chilense* and their deduced amino acid sequence. Our comparison of these sequences with those from *H. vulgare* will contribute to a better understanding of the genetic variability of the gamma-hordein family and may lead to better exploitation in breeding programs.

Materials and methods

Plant material

We used ten diploid accession lines of *Hordeum chilense* (Roem. et Schulz)—H1, H7, H11, H16, H47, H210, H220, H252, H293 and H297—from the germplasm collection of the Instituto de Agricultura Sostenible, CSIC, Cordoba, Spain in this study. The plants were grown in a greenhouse with supplementary lighting under a 12/12-h (day/night) regime.

Amplification of short fragments of gamma-3 hordein genes

Primers HoG*5 to HoG*8, which were used to amplify the short fragments of the *H. chilense* gamma-3 hordein, are described in Table 1. These primers were designed on the basis of the published sequence of the mRNA sequence of the gamma-3 hordein from *H. vulgare* (GenBank accession no. X72628). All of the primers used in this work were synthesized by Sigma-Genosys (Pampisford, UK).

DNA Isolation and PCR conditions

Genomic DNA from the *H. chilense* lines investigated was isolated using a CTAB method (Stacey and Isaac 1994). The PCR amplification of the short fragments of *H. chilense* gamma-3 hordein was carried out using 1× Gold Buffer, 400 μM dNTPs, 1.25 U *AmpliTaq* Gold, all obtained from Applied Biosystems (Foster City, Calif.), 100 ng of genomic DNA and 0.5 μM of one of the following primer combinations: HoG*5/HoG*6, HoG*7/HoG*8, HoG*5/HoG*8 and HoG*6/HoG*7. PCR amplification was run as follows: an initial step of 94°C for 10 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; a final step of 5 min at 72°C.

The resulting products were gel-purified, cloned in the pGEM-T Easy vector (Promega, Madison, Wis.), and transformed into competent *Escherichia coli* (DH5α) cells. The plasmid was purified using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, Calif.) and used as the template for sequencing. These sequences have been deposited at GenBank under accession numbers AY338379 to AY338385.

RNA isolation

RNA was isolated using the TRizol reagent (Invitrogen, Carlsbad, Calif.) according to the company's instructions and treated with DNase I (RNase-free) (Roche Diagnostics, Basel, Switzerland) to eliminate any DNA contamination. When the RNA was used for the synthesis of cDNA, one control PCR amplification was routinely performed to ensure that the specific PCR products were from RNA.

Rapid amplification of cDNA ends (RACE PCR)

Total RNA from the endosperm of *H. chilense* line H7 10 days after flowering was used to synthesize the anchored cDNA and the 3′ and 5′ ends as described by the SMART RACE cDNA amplification kit from BD Biosciences, (Palo Alto, Calif.). The specific primers HoG*9 and HoG*10 were designed using the short fragments previously sequenced to generate overlapping 5′- and 3′-RACE products. The 5′ and 3′ end products were size-fractionated by gel electrophoresis and the bands eluted using the Gel Extraction kit (QIAGEN), cloned, and then sequenced as described above. Corresponding accession numbers for these sequences are AY338371 to AY338378.

Full-length mRNA isolation

The sequences obtained from the 5′ and 3′ end products were used to design the primers HoG*19 and HoG*20 (Table 1), which amplify the full open reading frame (ORF) from the mRNA consensus sequence.

The cDNA was generated using total RNA from *H. chilense* line H1 using Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif.) according to the company's instructions. These full-length cDNA sequences were then ligated into the pGEM-T Easy vector (Promega) and sequenced. The accession numbers for these sequences are AY338365 to AY338370.

Quantitative real time-PCR (QRT-PCR)

Developing grains were harvested 4, 8, 12, and 18 days after flowering, which corresponds to growing stages nos. 69, 71, 75, and 83, respectively, according to Zadoks code (Zadoks 1974). Total RNA was extracted as described above. The synthesis of single-stranded cDNA was carried out using Superscript II reverse transcriptase (Invitrogen) and oligo $(dT)_{12–18}$ primers (Amersham Biosciences, UK). The real time quantitation of gene transcription

Table 1 PCR primers and their corresponding sequences used for cloning and characterization of the gamma-3 hordein from *Hordeum chilense*

Primer	Description	Sequence (5′→3′)
HoG*5	External forward for γ-3 hordein	GCCGCAACAACAATTTCCGCAGCA
HoG*6	External reverse for γ-3 hordein	TTTGCATGACACTTTATTTTTCATTGCTACATCGA
HoG*7	Nested forward for γ-3 hordein	CAAAACAGTTGCCAATTGAAGCGACAACAATG
HoG*8	Nested reverse for γ-3 hordein	GGGCTTGTTGTTGAGGTAGGACAAAGGCCTG
HoG*9	Specific primer for 3′ RACE-PCR	CACTGGCAAATATCAATGAGCAGTCCCGATG
HoG*10	Specific primer for 5′ RACE-PCR	GGAGCAATAAGGTGGGACATGCACGTTG
HoG*19	Forward for "edge-to-edge"	CATCCATCCTTCACAGATCTAGAGCACA
HoG*20	Reverse for "edge-to-edge"	GAATCGCACAGATGATGGTGCACGACA
HoG*13	Forward for QRT-PCR	CACAGCAATCATATCCGCAGTGGCAACG
HoG*14b	Reverse for QRT-PCR	GAAGTGGCATCTGCTGCGGAAATGGAC
HcITS*3	Forward for ITS1 ribosomal gene	CAGACCGCGCTCGTGTCATCCAA
HcITS*4	Reverse for ITS1 ribosomal gene	TGACCCCGAGTTAGGCACAGTGTTCCTTG

was performed using SYBR green staining on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the gene-specific primers HoG*13 and HoG*14b (Table 1). These primers were designed on the basis of the alignment with hordeins D, C and B from *H. chilense* (data not shown). A melting curve was used to identify the temperature at which only amplicons from H1 and H7 accounted for SYBR Green-bound fluorescence.

In QRT-PCR analysis, quantification is based on threshold cycle (C_t) values. The PCR signal is initially below the limit of detection and increases with cycle number to cross a threshold. The cycle number at which the signal crosses the threshold is defined as C_t and is inversely proportional to the logarithm of the initial copy number (Log CO). Each reaction was performed in triplicate and the corresponding C_t values were determined. The C_t values of each QRT-PCR reaction were normalized in relation to the C_t value corresponding to a *H. chilense* control gene, which was amplified using specific primers of the internal transcribed spacer (ITS1) of 18S-5.8S-26S ribosomal genes from *H. chilense* (GenBank accession no. AJ288116) using the primers HcITS*3 and HcITS*4 (Table 1). The efficiency of each particular QRT-PCR was also calculated. Subsequently, all of these values were utilized to determine the fold changes of gene transcription between the different grain developmental stages. The levels of gene transcription were processed and standardized according to Muller et al. (2002).

Bioinformatic analyses

The bioinformatic analyses were performed with LASERGENE 5.5 software from DNAStar (Madison, Wis.), OLIGO 6.83 software from Molecular Biology Insights (Cascade, Colo.) and SEQUENCHER 3.1.1 software from Gene Codes (Ann Arbor, Mich.). Sequence identity searches were performed at the National Center for Biotechnology Information (NCBI) via GENEQUEST 5.5 from DNAStar. The BLAST algorithms were run from the NCBI via the LASERGENE package. These applications were run on Mac OS ×10.2.6 from Apple Computer (Cupertino, Calif.). Signal peptides were determined using the SignalP engine at http://www.cbs.dtu.dk/services/SignalP.

Results and discussion

Isolation of the 3′ and 5′ ends and the full-length sequences of the gamma-3 hordein genes

Short fragments of 450, 500, and 700 bp were amplified using the primer pairs HoG*6/HoG*7, HoG*5/HoG*8, and HoG*5/HoG*6, respectively. The sequences of these fragments were first confirmed by BLASTN search as encoding gamma-3 hordeins, and then the sequences were used as templates for designing the oligonucleotides HoG*9 and HoG*10 to amplify the overlapping 3′ and 5′ ends of the gamma-3 hordein from cDNA of *H. chilense* line H7 by RACE-PCR. The 3′ and 5′ ends were then assembled; the full-length consensus sequence for the gamma-3 hordein is showed in Fig. 1. Primers HoG*19/HoG*20 (Table 1) were designed using this consensus sequence as a template to amplify the complete ORF of the gamma-3 hordein from cDNA of *H. chilense* line H1. Following sequencing, six different clones were identi-

Fig. 1 **A** Alignment of consensus sequences of the gamma-3 hordein from *Hordeum chilense* lines H1 (*upper*) and H7 (*lower*). The consensus sequences for lines H7 and H1 were obtained by RACE-PCR and edge-to-edge amplification of cDNA, respectively. Ambiguities are *shaded* (*R* purine, *Y* pyrimidine). **B** Single base ambiguities within the H1 and H7 sequences showing SNPs and GTC codon insertions between the consensus sequences of H1 and H7. Polymorphism positions are indicated based on the H1 sequence

A

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Line H1                                                                  1
CATCCATCCTTCACAGATCTAGAGCACAAACATCAAAACAAAACAATCAAGAGTACCCACAAATAAACCATGAAGATCTTCCTATTGTTTGCCCTCCTTG 31
CATCCATCCTTCACAGATCTAGAGCACAAACATCAAAACAAAACAATCAAGAGTACCCACAAATAAACCATGAAGATCTTCCTATTGTTTGCCCTCCTTG 31
Line H7

GTTTGGCAACAACCATCACTACCGCCACCGTGCAGTTCGACCCAAGCGGCCATGGCGTACAAAGGCCACAGCAATCATATCCGCAGTGGCAACCAGTTCC 131
GTTTGGCAACAACCATCACTACCGCCACCGTGCAGTTCGACCCAAGCGGCCATGGCGTACAAAGGCCACAGCAATCATATCCGCAGTGGCAACCAGTTCC 131

GCAACAACAACCATTTCCACAACAAGATCCACAACAACAACTACTTCCCCAACAACAACCATTTTCACAGCAACAACAACTTCCCCAGCAACATCCATTT 231
GCAACAACAACCATTTCCACAACAAGATCCACAACAACAACTACTTCCCCAACAACAGCCATTTTCACAGCAGCAACAACTTCCCCAGCAACATCCATTT 231

CCGCAGCAGATGCCACTTCTGCCGCAACCACCACAATTCCCGCAACAACAACCATTTGCCCAACCTCAACAACCACTAACACAACAACCATATCCGCAAG 331
CCGCAGCAGATGCCACTTCTGCCGCAACCACCACAATTCCCGCAGCAACAACCATTTGCCCAACCTCAACAACCACTAACACAACAACCATATCCGCAAG 331

AGCAACCATTGTCACAACAACAACCTTCTGTAGAGGAACAACAACAGTTGAACGTGTGCAAGGAGTTCCTCCTGCAGCAGTGCAACCCGAACGAGAAGGT 431
AGCAACCATTGTCACAACAACAACCTTCTGTAGAGCAACAACAACAGTTGAACGTGTGCAAGGAGTTCCTCCTACAGCAGTGCAACCCGAACGAGAAGGT 431

ATCGTCACTCCAGTCAGTGATCCCTTTCCTTCGTCCACAGACCTGGCAACAGAACAGTTGCCAATTGAAGCGACAACAATGTTGTCGACAACTGGCAAAT 531
ATCGTCACTCCAGTCAGTGATCCCTTTCCTTCGTCCACAGACCTGGCAACAGAACAGTTGCCAATTGAAGCGACAACAATGTTGTCGACAACTGGCAAAT 531

ATCAATGAGCAGTCCCGATGCCCAGCCATCCAAACCATCGTGCACGCCAYCGTCATGCAACAACAACAAGTACAACAACAGGTGGGTCATGGTTTTATCC 631
ATTAATGAGCAGTCCCGATGCCCAGCCATCCAAACCATCGTGCACGCCATCGTCATGCAACAACAACAAGTACAACAACAGGTGGGTCATGGTTTTATCC 631

AATCTCAACCACAACAGTTGGGCCAAGGAATGCCCATCTACCCTCRACAYCAACCGGGCCATGGCTTTTTTCTACCTCAACAACAAGCCCAGTCGTTCAA 731
AATCTCAACCACRACAGTTGGGCCAAGGAATGCCCATCTACCCTCAACAACAACTGGGCCATGGCYTTTTTCTACCTCAACAACAAGCCCA----GTTCAA 728

CTTGGTTAGGTCATTGGTGATTCAGACACTACCTATGTTATGCAACGTGCATGTCCCACCTTATTGCTCCACCACTACTGCACCATTTGGTAGCATGCCT 831
CTTGGTTAGGTCATTGGTGATTCAGACACTACCTATGTTATGCAACGTGCACGTCCCACCTTATTGCTCCACCACTACTGCACCATTTGGTAGCATGCCT 828

ACCGGCATCRGTGGCCAATGAAATGACAAGAGTTGTAATACTAGATAGATGGACCTTCATTGTTAGTCGATTGGRGGATCGATGTAGCAATGAAAAATAA 931
ACCGGCATCGGTGGCCAATGAAATGACAAGAGTTGTAATACTAGATAGATGGACCTTCATTGTTAGTCGATTGGAGGATCGATGTAGCAATGAAAAATAA 928

AGTGTCGTGCACCATCATCTGTGCGATTC 960
AGTGTCGTGCACCATCATCTGTGCGATTCCAGTCAGTACTAGTTCAAACATGGGAATAAAGACAAACAATTATTGTTTGCATAAAAAAAAAAAAAAAAAA 1028

AAAAAAAAAAAAA 1045
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B

Line	Polymorphism position																	
	-20	189	204	276	367	405	504	534	581	644	651	677	681	686	697	723	783	841	906
H1	A/G	A	A	A	G	G	A	C	T/C	A	A/G	A/G	A/T	C	T	GTC	T	G/A	G/A
H7	A	G	G	G	C	A	A/G	T	T	A/G	G	A	A	T	T/C	---	C	G	A

fied, and the edge-to-edge consensus sequence was aligned with that from line H7 (Fig. 1).

Nucleotide sequence analysis

BLASTN searches of both consensus sequences confirmed the similarity with the gamma-3 hordein gene from *H. vulgare*. The ORFs for H7 and H1 contain 849 bp and 852 bp, respectively. Small differences involving single base changes were observed within both consensus sequences (Fig. 1A). For H7, three single ambiguities are present at positions 504, 644 (A or G) and 697 (T or C) (Fig. 1B). For H1, single ambiguities are present at positions –20, 651, 677, 841, 906 (A or G), 581 (T or C), and 681 (A or T) (Fig. 1B). Enzymes with proof-reading activity were used for the amplification, and three different clones were sequenced in both forward and reverse directions to minimize the possibility of PCR errors. Consequently, ambiguities within the sequence from each line could be explained on the basis that several copies of the gene are present at the same locus, as described previously for the B hordein of *H. vulgare*, for which 15–30 copies of the gene are present at the *Hor2* locus (Kanazin et al. 1993). Between H1 and H7, differences involving single base changes at positions 189, 204, 276, 367, 405, 534, 686, and 783 and the insertion of the codon GTC at position 723 (Fig. 1B) could correspond to single nucleotide polymorphism (SNPs). It is interesting to note that only two of these SNPs cause changes in amino acid residues: the changes of C (glutamine in H7) for G (glutamic acid in H1) and T (phenylalanine in H7) for C (leucine in H1) at positions 367 and 686, respectively. In wheat, SNPs for the gamma-gliadins, which are homologous to gamma-hordeins, have been recently reported (Zhang et al. 2003). However, the occurrence of SNPs between these two lines of *H. chilense*— about one SNP per 100 bp—is much higher than that of wheat—one SNP per kilobase (Zhang et al. 2003).

The ATG starting translation codon is not preceded with the GCCACC consensus sequence of some eukaryotic genes (Kozak 2001), indicating that the first methionine residue may not belong to the final protein. The 5′ untranslated region (leader sequence) contains 70 bases and lacks the AAAGA sequence, implicated in the secondary structure of the mRNA, which is found in some eukaryotic mRNAs. Likewise, no stop codon is present immediately preceding the coding sequence (CDS) but at –6 upstream (TAA; opal) from the ATG ORF codon start. On the other hand, the 3′ untranslated region shows four extra stop codons in frame (TAG, TAG, TAG and TAG). In common with the 3′ untranslated regions of most plant genes (Dean et al. 1986) this gamma-3 hordein has two polyadenylation signals (AATAAA) at positions 75 and 130 downstream of the ORF stop codon (Fig. 1A).

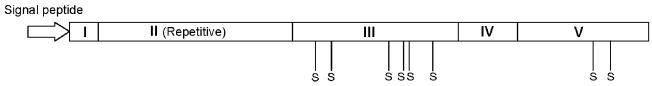

Fig. 2 Schematic structure of the gamma-3 hordein from *H. chilense*. The signal peptide and different regions are indicated. *S* denotes the positions of the cysteine residues

Amino acid sequence analysis

The translated precursor proteins of the gamma-3 hordeins from *H. chilense* contain 282 and 283 amino acid residues for lines H7 and H1, respectively. The BLASP of both amino acid sequences confirmed higher homology with the gamma-3 hordein, gamma-gliadins and avenin-3 from cultivated barley, wheat and oat, respectively. The alignment with those sequences is shown in Fig. 2. The gamma-3 hordein polypeptides from *H. chilense* have similar features to other S-rich prolamins, and specifically to those described for gamma-gliadins (Shewry et al. 1995; Anderson et al. 2001): there is a core repetitive region flanked by two non-repetitive regions, with eight cysteine residues which form intramolecular disulfide linkages (Fig. 2).

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 (MKIFLLFALLGLATTITTA). Of the 19 amino acid residues, 14 are hydrophobic and four are polar residues just before the cleavage site, which is a common feature in the signal peptide of most prolamins (Kreis and Shewry 1989). To our knowledge, this is the first signal peptide reported for a gamma-3 hordein. This leader peptide is similar to that for the B1 hordein of *H. vulgare* (MKTFLIFALLAIAATSTIA) (Brandt et al. 1985; Forde et al. 1985). The signal peptide is flanked by the dipeptide MK at the N-terminal end and by the dipeptide TA at the C-terminal end, as reported for gamma-gliadins (Fig. 3) (Anderson et al. 2001). It is followed by a short region of 13 amino acid residues (N-terminal region), which is a common feature of most prolamins (Kreis and Shewry 1989). This short region is similar to that of the gamma-gliadins of wheat and shows great divergence in the carboxyl terminal region with respect to the gamma-3 hordein from cultivated barley (Fig. 3). On the other hand, the N-terminal non-repetitive region is followed by a repetitive region of 87 residues (see below).

The region between the repetitive and the carboxyl-terminus regions of the gamma-3 hordein is poor in proline and rich in cysteine and methionine, having stretches of polyglutamine in some regions. Anderson et al. (2001) have distinguished three regions for the gamma-gliadins: a region containing most of the cysteine residues (III), another which is rich in glutamine residues (IV), and the C-terminal non-repetitive region that contains two additional conserved cysteine residues (V) (Fig. 3). These three regions can also be observed in the amino acid sequence of the gamma-3 hordein from *H. chilense*. However, region III is longer in both *H. chilense*

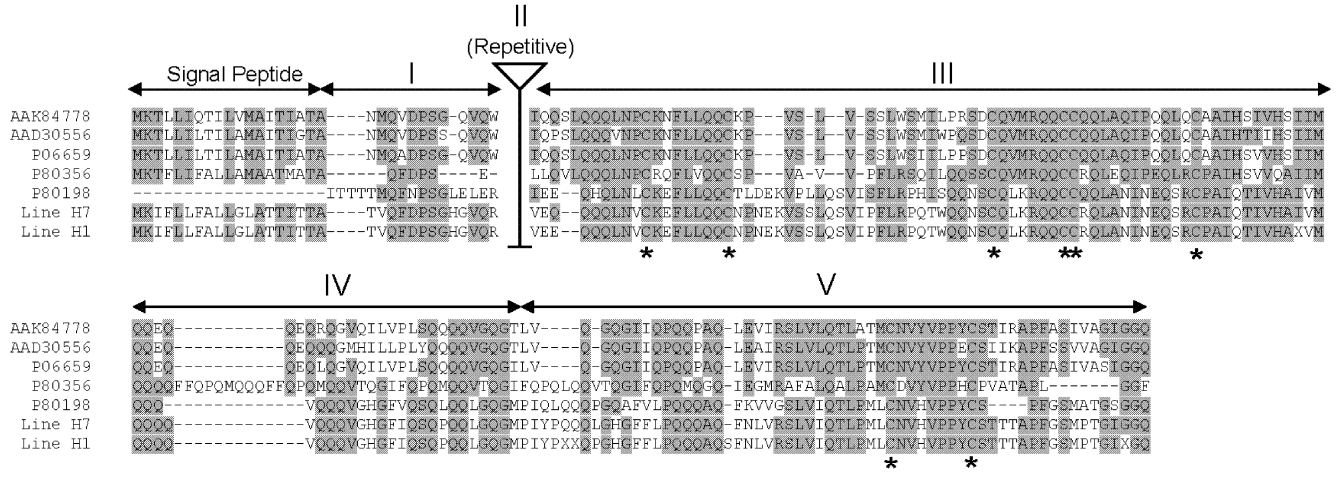

Fig. 3 Alignment of the gamma-3 hordein sequences from *H. chilense* lines H7 and H1 with the gamma-gliadins from wheat (*AAK84778*, *AAD30556*, and *P06659*), avenin-3 from oat (*P80356*), and gamma-3 hordein from *H. vulgare* (*P80198*). The polypeptide regions are as for gamma-gliadins in Anderson et al. (2001). *Vertical bar* indicates the position of the repetitive region (removed for this analysis); *asterisks* indicate the positions of the eight cysteine residues. The more conserved sequences are indicated in *gray*

and cultivated barley than in the gamma-gliadins of wheat. The positions of six residues of cysteine and the flanking sequences are well conserved in all sequences of gamma-3 hordeins, gamma-gliadins and avenin-3 of oats (Fig. 3). Region IV is rich in glutamine in both the H1 and H7 lines: 12 out of 25 residues. These glutamines are present mainly in short runs of two to four residues, suggesting a DNA slip-mismatching of glutamine codons during replication (Cassidy et al. 1998). Polyglutamine stretches are a common feature in α- and in gamma-gliadins (Rafalski 1986; Anderson and Greene 1997) and LMW-glutenins (Cassidy et al. 1998). Region V contains two cysteine residues, and it is highly conserved in the C-terminal end in all gamma-3 hordeins, gamma-gliadins and avenin-3 (Fig. 3).

Repeat structure

The nucleotide and deduced amino acid sequences of the repeat region of the gamma-3 hordein from *H. chilense* are shown in Fig. 4A to emphasize the motif structure. The DNA consensus sequence, based on the most frequently occurring nucleotide for each position in the codon, is CCR CAA CAA CAA CCA TTT, and its corresponding amino acid motif is PQQQPF. Variations of this consensus sequence within the repetitive region are usually due to single-pair changes (Fig. 4A). The repetitive region of a gamma-3 hordein of *H. vulgare* (accession no. X72628) is also shown in Fig. 4B. While both of these gamma-3 hordeins share the same repetitive motif, in the one from *H. vulgare* the repetitive region consists of 19 repeats, whereas that from *H. chilense* is made up of 15 tandem repeats. In wheat, variation in the repetitive region is well documented in gamma-gliadin and other prolamins. Our observation could indicate that the process

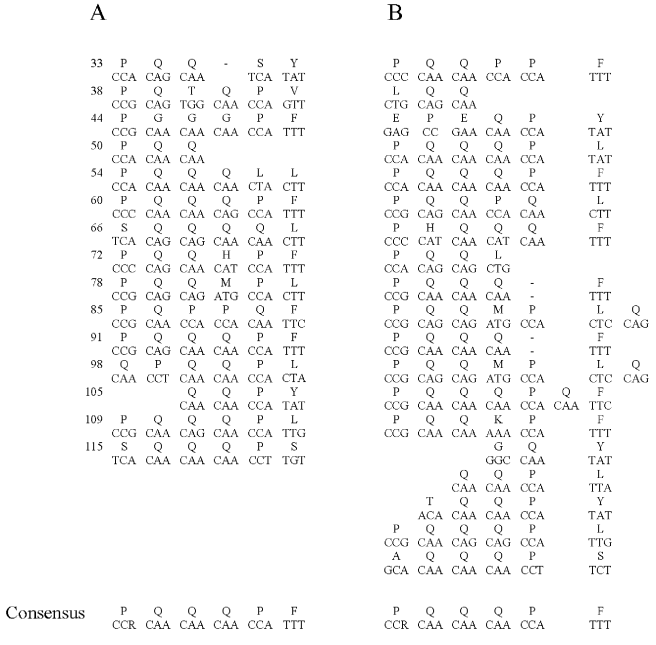

Fig. 4 Alignment of the repetitive motif for the gamma-3 hordein from both *H. chilense* (**A**) and *H. vulgare* (**B**). The alignment is based on the most frequently occurring nucleotide for each position. The consensus codon repeats and the derived amino acid residue patterns are given (*below*)

of shrinking and expansion of the repetitive region may also occur in the barley genome. Variations in the length of the repetitive region of the B hordein gene between cultivated and wild genotypes of barley were also reported by Kanazin et al. (1993).

The tandem repeat motif of gamma-3 hordein from *H. chilense* is similar to those reported for the gamma-gliadins QPQQPFP (Scheets et al. 1985; D' Ovidio et al.

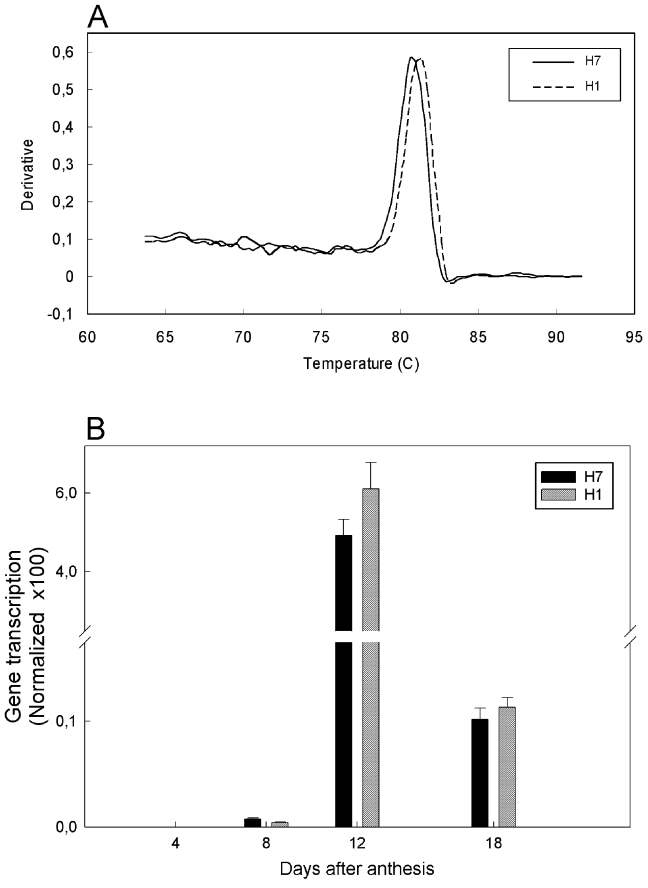

Fig. 5A, B Analysis by QRT-PCR of the transcription of the gamma-3 hordein gene from H7 and H1 lines in the developing endosperm. **A** Dissociation curves to determine the melting temperature of both H7 and H1 gamma-3 hordein genes using the HoG*13 and HoG*14b primers, respectively. **B** The fold changes in gene transcription was determined at different grain developmental stages, normalized after Muller et al. (2002), and represented in arbitrary units

1995), PQQPF plus PQQPQQ(Q)PFP (Rafalski 1986), and $PFPQ_{1-2}(PQQ)_{1-2}$ (Anderson et al. 2001). In most cases, the difference between the motif of the *H. chilense* gamma-3 hordein and those of others prolamins is the addition of the PQQ tripeptide (one or two repeats) at the end of the motif. This is evident in the gamma-secalin and C hordein, which has the sequence PQQPFPQQ (Tatham and Shewry 1995) and for the gamma-gliadin, with the sequence $PFPQ_{1-2}PQQ$ (Hsia and Anderson 2001). The gain of three codons by duplication seems to be a common phenomenon in the evolutionary processes of these repetitive regions (Anderson and Greene 1989).

Storage proteins are expressed coordinately in the developing endosperm. The transcription of the gamma-3 hordein genes was monitored by QRT-PCR (Fig. 5), which is a highly sensitive technique used to detect small amounts of mRNA. As the control gene, we used the internal transcribed spacer (ITS1) of 18S-5.8S-26S ribosomal genes from *H. chilense*. The ITS1 has been successfully used as the control gene in other genotypes (Benítez-Burraco et al. 2003). The efficiency of both the target and control genes was similar in each QRT-PCR. Dissociation curves for the H7 and H1 gamma-3 hordein genes are shown in Fig. 5A. The difference in melting temperature between both sequences can be explained on the basis of two SNPs in the region amplified by the HoG*13 and HoG14b primers. As expected, the transcription of this gene was limited to the developing grain, and no transcription was found in roots and leaves (data not shown). In the endosperm of both lines, the transcription of this gene began 8 days after flowering and reached a maximum 12 days after flowering (Fig. 5B). Cameron-Mills and Brandt (1988) reported that mRNA for a gamma-1 hordein in common barley was detected between 8 days and 26 days after anthesis. Gamma-3 hordein is a monomeric protein that only forms intramolecular disulfide bridges, and the role of this storage protein in the formation of the complex protein or its transport is not well understood. Rechinger et al. (1993) have proposed that the gamma-3 hordein may play an important role in maintaining the prolamin storage polypeptides in a transport-competent state. In this way, overexpression or silencing of this gene in barley and wheat, by means of antisense technology, may be useful means of elucidating the function of this gene and, hence, of gaining a further understanding of storage protein deposition in the developing grain.

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