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Evolutionary relationship of the members of the sulphur-rich hordein family revealed by common antigenic determinants

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Summary. Five monoclonal antibodies raised against an enriched C hordein fraction have been characterized in detail and were found to be specific for the members of the sulphur-rich hordein family. Two antibodies specific for B hordein polypeptides were identified, one of which reacted predominantly with CNBr cleavage class III polypeptides. $\gamma 1$ hordein was recognized by two antibodies, of which one also reacted with $\gamma 2$ hordein and several members of the CNBr cleavage class II B hordein polypeptides. One antibody recognized $\gamma 3$ hordein but cross-reacted at higher antibody concentration with almost all of the B and C hordein polypeptides. The specificity of the monoclonal antibodies was confirmed by Western blotting of one- or two-dimensionally separated hordein from the B hordein-deficient mutant *hor2ca* and its wild-type Carlsberg II and the $\gamma 3$ hordein-deficient genotype Nevsky. The identification of the γ hordein-specific monoclonal antibodies was further supported by immune precipitation of in-vitro transcribed and translated $\gamma 2$ hordein, and *hor2ca* and Carlsberg II mRNA translation products. The monoclonal antibodies were used to screen for mutants in γ hordein synthesis. Two mutants, one deficient in $\gamma 1$ hordein synthesis and a second in $\gamma 2$ or closely related B hordein polypeptides were identified. A model is proposed for the evolution of the sulphur-rich hordein loci *Hor5* and *Hor2*.

Key words: Barley – Monoclonal antibodies – γ hordein mutant – Prolamin – Two-dimensional gel electrophoresis

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Introduction

Hordein, the prolamin fraction of barley, is the major class of storage protein in the endosperm tissue of mature grains. It accounts for approximately 50% of the total protein and consists of heterogeneous groups, namely D (high molecular weight), C (sulphur-poor), B and γ (both sulphur-rich) hordein polypeptides. B and C hordein are the major groups and comprise up to 95% of the total hordein fraction. Common features of barley prolamins are extreme hydrophobicity, solubility in isopropanol, low content of charged amino acids, notably the essential amino acid lysine, and elevated levels of proline and glutamine. The primary structure of C hordein polypeptides, deduced from their genes, is composed of tandemly repeated octapeptides rich in proline and glutamine (Entwistle et al. 1991). This repeat structure comprises the N-terminal domain of B and γ hordein-deduced protein sequences, while the C-terminal domain contains the majority of the sulphur amino acids and only a few degenerated repeats (Brandt et al. 1985; Cameron-Mills and Brandt 1988).

The different hordein groups are coordinately expressed during endosperm development (Sørensen et al. 1989; Giese et al. 1983). Hordein polypeptides are synthesized on membrane-bound polysomes, with co-translational cleavage of the signal peptide and concomitant transport of the newly synthesized polypeptides into the lumen of the ER (Cameron-Mills and Madrid 1989). The prolamin polypeptides are subsequently deposited in the vacuole of the endosperm cell, where they aggregate into a fibrillar matrix, which is a transient stage in the condensation of storage proteins into homogeneous, electron-dense structures named protein bodies (Cameron-Mills 1980; Cameron-

Mills and von Wettstein 1980). In the absence of any functional constraints on hordein polypeptide sequence, it is likely that the conserved features of hordein are related to protein body formation and the packing and disulphide cross-linking of the polypeptide chains. Barley mutants largely depleted in the total amount of B and C hordein, are not reduced in plant viability, but form less-condensed protein aggregates (Cameron-Mills and von Wettstein 1980).

The multigene families encoding the C, B, D and γ hordein polypeptides have been assigned to chromosome 5 at the loci *Hor1*, *Hor2*, *Hor3* and *Hor5*, respectively (Jensen et al. 1980; Shewry et al. 1980; Blake et al. 1982; Shewry et al. 1983; Shewry and Parmar 1987). The *Hor2* locus comprises two subloci (Sørensen et al. 1989; Shewry et al. 1990) and maps close to *Hor5*, which also encodes S-rich hordein. Although cDNAs and genomic clones representing one to two members of each family are available, the polymorphism within hordein groups and the homology between families have not been clearly assessed. The analysis of sequence diversity within a single family is hindered by a general cross-hybridization of the gene probes of hordein-coding sequences with all members of a family.

This paper analyzes the structural relationship between polypeptides of the B and γ hordein family through the use of monoclonal antibodies. Five monoclonal antibodies, raised against an enriched C hordein fraction of cv Bomi by Ullrich et al. (1986) have been characterized in detail. To identify the exact target of each antibody, well defined hordein-deficient mutants were analyzed. In particular *hor2ca*, a mutant derived from cv Carlsberg II (Doll 1980) and depleted of all B hordein polypeptides, enabled the identification of γ1-, γ2-, and γ3-type hordeins in barley, which are otherwise masked by their co-migration with B hordein polypeptides in SDS-PAGE (Kreis et al. 1983b; Shewry et al. 1985; Cameron-Mills and Brandt 1988). This paper describes new γ hordein mutants, which lack specific polypeptides and can be used to map the γ hordein genes. We present a model of the evolutionary relationship of the members of the sulphur-rich prolamin family in barley.

Materials and methods

Plant material

Barley plants (*Hordeum vulgare* L.) cv Carlsberg II and its B hordein deletion-mutant *hor2ca* (Doll 1980), and cv Bomi and its high-lysine mutant *lys3a* (Karlson 1977), were grown in the greenhouse or field. Grains of Krasnoufimsky 95, Donetsky 8, Omsky 80, Kharkovsky 70, Nutans 115 and Nevsky, which lacks a *HrdF*-encoded polypeptide when analyzed by starch-gel electrophoresis (Pomortsev, personal communication), were kindly provided by A. A. Pomortsev, Institute of General Genetics, Moscow, Russia. Grains of Weihenstephan CP 127422 and Haisa were kindly provided by V. P. Netsvetaev, Institute of Plant Breeding and Genetics, Odessa, Ukraine.

Monoclonal hordein antibodies

Protein A-purified monoclonal mouse antibodies (Ullrich et al. 1986) BI (CMpBhorI), BII (CMpBhorII), BX (CMpBXhor), X (CMpXhor) and BC (CMpBChor) were stored at −20 °C in aliquots, at a protein concentration of 0.2 mg/ml (OD_{280}). Once thawed, they could be kept for several weeks at 4 °C, without any decrease of sensitivity or loss of specificity.

Hordein extraction

Single, mature kernels were crushed and total hordein was extracted with 200 µl of 55% isopropanol, 2% β-mercaptoethanol at 60 °C for 1 h with occasional shaking. Following centrifugation for 5 min at 12,000 g, 800 µl of water was added to the supernatant and the mixture cooled in liquid nitrogen. After thawing, the hordein precipitate was recovered by centrifugation (5 min, 12,000 g), freeze-dried and stored at −20 °C.

Pyridylethylation

Hordein was dissolved at a concentration of 10 mg/ml in 6 M urea, 2% β-mercaptoethanol and 10 mM Tris-HCl pH 7.5 buffer. Oxygen was removed by flushing with N_2 and 130 µl/ml of 2-vinylpyridine (not 4-vinylpyridine) was added (Friedman et al. 1970). The reaction was stopped after 2 h by lowering the pH to 2–2.5 with concentrated acetic acid. The pyridylethylated hordein was dialyzed overnight against 0.01 M acetic acid and freeze dried.

SDS-PAGE and Western blotting

Electrophoresis and Western blotting were performed with the Bio-Rad (Richmond, USA) Mini-PROTEAN II system, using 12% polyacrylamide gels (Laemmli 1970). Samples were dissolved in a buffer containing 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.00125% bromophenol blue and boiled for 1 min prior to loading. Electrophoresis was stopped when the green 21.5 kDa Rainbow molecular weight marker (Amersham, UK) reached the end of the gel. After 15 min equilibration in transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH) pairs of gels were electro-blotted for $1\frac{1}{2}$ h at 110 V, 150 mA (starting conditions) onto a 45 µm pore-size nitrocellulose membrane (Millipore, Bedford, USA). The coloured markers were then visible on the membrane. Staining the gels after transfer with Coomassie Blue, showed that the smaller reference proteins had disappeared, although enough protein remained to visualize the characteristic hordein pattern.

Coomassie Blue staining

Gels were stained [0.03% Coomassie Brilliant Blue R250, 10% trichloracetic acid (TCA), 40% MeOH] for 1 h and destained in 40% MeOH, 10% TCA followed by 10% TCA alone.

Two-dimensional (2D) gel electrophoresis

The 2D gel electrophoresis system of Shewry et al. (1988), which is based on Righetti et al. (1977), was adapted to the Mini-PROTEAN II system.

First dimension. One and a half millimeter isoelectric focussing (IEF) gels [acrylamide/bis (4.6% T, 4.76% C), 0.05% ammoniumpersulphate, 5.6% 2D Pharmalyte pH 3–10 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and 6 M urea] were cast onto an FMC-Gelbond polyacrylamide gel-support medium (FMC, Rockland, USA). Pieces of teflon glued to one glass plate formed 3 × 9 × 0.75 mm-sized sample wells. IEF was performed at 12 °C on a Multiphor II apparatus (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with electrodes placed at a distance of 6.5 cm. Wicks were soaked in 0.5 M acetic acid (+) and 0.5 M NaOH (−). Gels were placed with the sample wells on the anodal side and pre-run for 40 min with 10 µl 6.25% 2D Pharmalyte pH 3–10 at 2.5 W. Wells were loaded with 10 µl of pyridylethylated-hordein [2 mg/ml in 10 mM glycine, 6 M urea, pH 8 (Tris)] and 5 µl FMC IEF pI markers pH 3–10 (FMC, Rockland, USA). IEF was performed at 7 W and stopped 15 min after the coloured marker focussed next to the anode. After trimming off the anodal and cathodal ends of the gel, two strips per well were cut out and either equilibrated for 30 min in SDS-PAGE stacking-buffer or fixed in 35% MeOH, 13% TCA and 3.35% sulphosalicylic acid and stained with Coomassie Blue.

Second dimension. A 1.5 mm-thick 12% Laemmli gel with a 1 cm stacking-gel was cast with a large well for the IEF strip and two small reference wells. These were loaded with either pyridylethylated hordein from Carlsberg II or *hor2ca*, dissolved in the IEF buffer, or with Rainbow molecular weight markers (Amersham, UK). SDS-PAGE and Western blotting followed as described above. Gels were subsequently stained, dried between cellophane and used to align the Westerns.

Immune assays

Immune assays were performed at 37 °C with a reaction volume of 15 ml. The membranes were blocked with 1% skim milk powder/phosphate buffered saline (PBS) for 20 min before a $1\frac{1}{2}$ h incubation with the primary antibody, diluted 1:2000, 1:200, 1:200, 1:200, 1:3000 for the BI, BII, BX, X and BC antibodies, respectively. Three washes with PBST (PBS + 0.5% Tween 20) were followed by 1 h incubation with peroxidase (P 161)- or alkaline phosphatase (D 314)-conjugated rabbit anti-mouse antibody (Dako A/S, Glostrup, Denmark), diluted 1:500 and 1:2000, respectively. After three washes with PBST (Tris buffered saline + 0.5% Tween 20 for alkaline phosphatase conjugates), colour assays were performed with either 3-amino-9-ethylcarbazole (peroxidase) or 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (alkaline phosphatase). Reactions were stopped by washing with tap water and the membranes dried.

Isolation and translation of poly A^+RNA

Spikes of Carlsberg II and *hor2ca* were harvested 20 days after anthesis, the endosperms isolated and immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was prepared from the developing endosperms according to Aviv and Leder (1972) with minor modifications. The frozen ground tissue was extracted in 0.1 M Tris-HCl pH 9, 10 mM EDTA, 25 mM dithiothreitol (DTT) and 1% SDS together with an equal volume of phenol, and the RNA precipitated from the aqueous phase with 2M LiCl at 4 °C. Poly A^+RNA was isolated from the RNA preparation by Poly(U)-Sepharose 4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) affinity chromatography and translated in a wheat germ extract (Roberts and Patterson 1974), as described by Cameron-Mills and Madrid (1989).

Construction of a γ hordein in-vitro expression plasmid

The coding regions of λhorγ-1 were cloned into the pDS6 expression plasmid (Stueber et al. 1984) using a PCR protocol according to Perkin Elmer Cetus (Vatterstetten, Germany). The template, a pBR328horγ-1 subclone, was annealed with a 31-b upstream primer between −23 and −1, and a 37-b downstream primer annealing at +320 to +338. The two primers contained 5′*Eco*RI and *Hin*dIII restriction sites, respectively, to facilitate cloning of the amplified coding region into the pDS6 polylinker, to give pDS6horγ-1. This construct was transformed into *E. coli* DH5α (Hanahan 1983) and propagated on ampicillin-containing medium. Plasmid DNA was prepared according to the Qiagen protocol (Diagen GmbH, Düsseldorf, Germany).

Coupled transcription and translation of the γ1 hordein gene

pDS6horγ-1 was transcribed with *E. coli* RNA polymerase in the presence of 7mGpppA to produce capped mRNA which was subsequently translated in the wheat germ system (Roberts and Patterson 1974) as described by Stueber et al. (1984). The polypeptides were synthesized with 20 µCi ^{35}S-methionine (43.88 TBq/mmol) per 25 µl assay. The translation products (3 µl aliquots) were analyzed by SDS-PAGE. The gels were subsequently fixed and dried for fluorography (Chamberlain 1979).

Immune precipitation of hordein polypeptides from cell-free translations

Polyclonal rabbit IgG antibodies raised against hordein from *H. vulgare* cv Bomi by Dierks-Ventling and Cozens (1982) were a gift. Immune precipitation of hordein polypeptides from 10 µl of poly A^+RNA translations, or 10 µl of the coupled transcription-translation products of pDS6horγ-1, was performed according to Anderson and Blobel (1983). Antigens were immune-precipitated overnight at 4 °C with either 50 µg of anti-hordein polyclonal IgG or 10 µl of hordein monoclonal antibody in a volume of 65 µl. The antibodies were coupled to Protein A-Sepharose beads and subsequently released by boiling in 20 µl of 125 mM Tris-BCl pH 6.8, 2.5% SDS, 10% glycerol and 50 mM DTT and then acetylated with 70 mM iodoacetamide at 37 °C. Immune precipitates were analyzed by SDS-PAGE as described above.

Results

Antibody recognition of hordein polypeptides separated by SDS-PAGE

The well-established hordein polypeptide composition of the genotypes Carlsberg II, its *Hor2* deletion-mutant *hor2ca*, Nutans 115, and Nevsky, was used to characterize the antibody specificity. Nevsky, while having a similar B hordein composition as Nutans 115, lacks a *HrdF*-encoded polypeptide. Western blots of hordein extracts from the four barley genotypes separated by SDS-PAGE were tested with the five hordein antibodies (Fig. 1A, B).

The BI and BII antibodies reacted with polypeptides migrating with the B hordeins. Since the polypeptides recognized by BI and BII were not present in *hor2ca*, they are considered to be B hordein

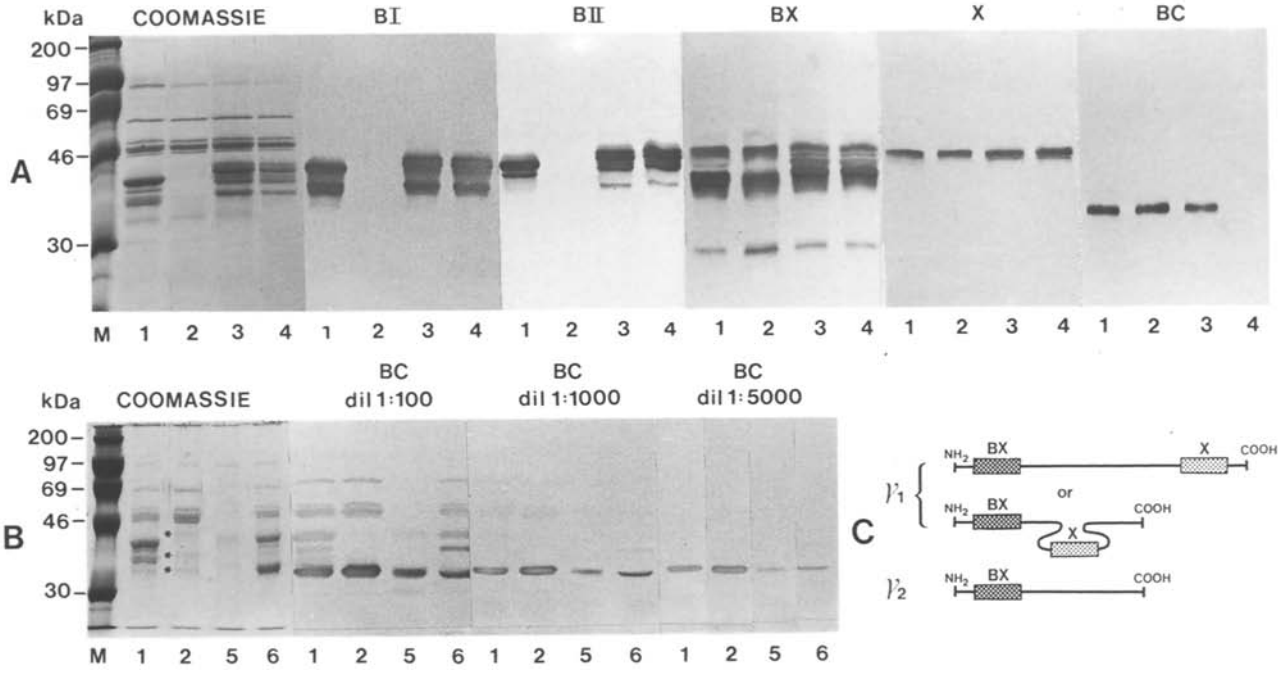

Fig. 1. Single grains of six barley genotypes (*1*, Carlsberg II; *2*, *hor2ca*; *3*, Nutans 115; *4*, Nevsky; *5*, *lys3a*; *6*, Bomi) were extracted in 55% isopropanol, 2% β-mercaptoethanol, and the hordein extracts analyzed by SDS-PAGE, Coomassie Blue staining **(A, B)**. **A** From replicate gels, Western blots were prepared and assayed with the monoclonal antibodies BI, BII, BX, X and BC. **B** The BC antibody was tested at three different dilutions. The *asterix* (*) indicates the position of the γ1, γ2 and γ3 hordein polypeptides in order of decreasing molecular weight. **C** A model is proposed for the location of epitopes on the γ1 and γ2 hordein polypeptides recognized by the BX and X antibodies

antibodies. Nutans 115 and Nevsky had a similar BI and BII antibody recognition pattern, in accordance with their similar B hordein composition. It seems likely that some of the hordein polypeptides contain discrete epitopes recognized by BI and BII antibodies. The BX antibody recognized at least two broad, diffuse, bands of 41 and 36 kDa and a minor polypeptide of 28 kDa in all four varieties. From the size of these polypeptides and their presence in *hor2ca*, the BX antibody is assumed to recognize γ hordein. Carlsberg II, Nutans 115 and Nevsky have additional polypeptides between 39 and 43 kDa recognized by BX. The X antibody reacted with a singlet or doublet of 41 kDa, present in all four varieties. The presence of these polypeptides in *hor2ca* suggests that they are also γ hordein.

Dilution series were performed with all five antibodies but only the BC antibody gave a variable antigen specificity (Fig. 1B). At high antibody concentrations B and C hordein were recognized, but at reduced concentrations, strong specificity towards a single 32-kDa band was revealed. Since this small hordein polypeptide was present in all the varieties tested, except Nevsky, it is probably the γ3 hordein encoded by the *Hor5* locus.

Antibody recognition of in-vitro synthesized hordein polypeptides by immune precipitation

Poly A^+RNA isolated from developing endosperm tissue of Carlsberg II and *hor2ca* was translated in a wheat germ system with ^{35}S-methionine. Immune precipitation with each antibody was then performed with Protein A-Sepharose. A polyclonal antibody (P), raised against a total hordein extract, was used to immune-precipitate all the hordein synthesized and provide a marker. The precipitated hordein polypeptides were analysed by SDS-PAGE and fluorography (Fig. 2A, B and C).

All five monoclonal antibodies immune-precipitated specific in-vitro synthesized polypeptides. BI and BII antibodies only precipitated polypeptides synthesized from Carlsberg II mRNA. The BII antibody specifically recognized the largest polypeptides in the B hordein region. One strong band, co-migrating with the smallest of the BX-precipitated polypeptides, was obtained with the BC antibody. X reacts weakly under the stringent conditions of the immune assay and polypeptides are only detected in the *hor2ca* mRNA translations. When the stringency is lowered by reducing the SDS concentration of the solubilized

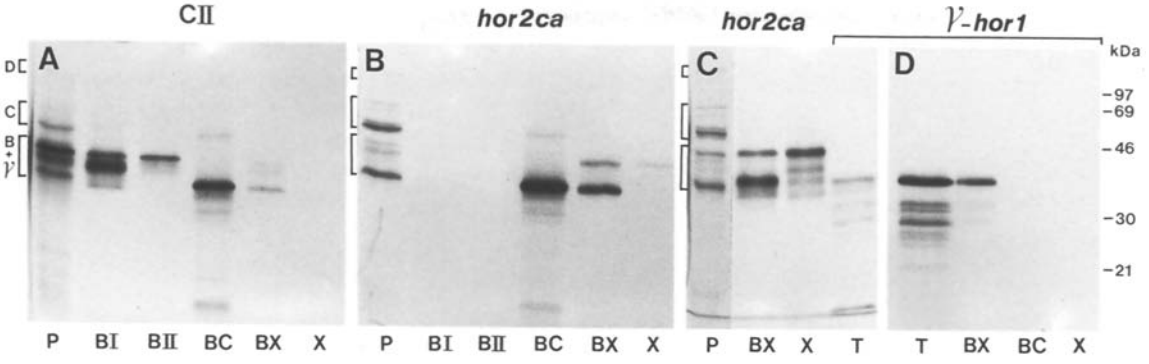

Fig. 2. Immune precipitates of ^{35}S-methionine-labelled in-vitro translation products of poly A$^+$RNA of Carlsberg II and *hor2ca* are compared to the products obtained by coupled transcription-translation of the expression vector pDS6horγ-1, and separated by SDS-PAGE and fluorography. **A** and **B** Carlsberg II and *hor2ca* in-vitro translation products were precipitated with a polyclonal antibody (P) and the monoclonal antibodies BI, BII, BC, BX and X. **C** *hor2ca* in-vitro translation products precipitated by P, BX and X at a lower stringency (0.1 instead of 0.2% SDS). T, polypeptides expressed by the vector pDS6horγ-1. **D** Polypeptides expressed from the vector pDS6horγ-1 were immune-precipitated with the BX, BC and X antibodies. T, polypeptides expressed by the vector pDS6horγ-1

translation products from 0.2% to 0.1%, the X antibody precipitation is enhanced. (Fig. 2B, C).

The genomic clone λhorγ-1 encodes a polypeptide whose sequence aligns with the N-terminal sequence of γ1 and γ2 hordein. The gene has been cloned in the expression vector pDS6, and transcribed and translated in a wheat germ system with ^{35}S-methionine. Immune precipitations were performed with BX, X and BC antibodies (Fig. 2D).

λhorγ-1 encodes a γ2 hordein polypeptide on the basis of its deduced protein sequence and its mobility in SDS-PAGE (Fig. 2C). This γ2 hordein polypeptide co-migrates with a 38-kDa polypeptide immune-precipitated by BX. The γ2 hordein polypeptide is immune-precipitated by BX, but not X or BC, antibodies (Fig. 2D).

The effects of pyridylethylation on the antigenicity of hordein polypeptides and their electrophoretic mobility

In order to maintain the SH groups of the cysteines in the hordein polypeptides in a reduced state during 2D separation (IEF + SDS-PAGE), pyridylethylation was performed with 2-vinylpyridine. Comparison of pyridylethylated and non-pyridylethylated hordein on Coomassie Blue-stained gels from Carlsberg II and *hor2ca* (Fig. 3A, B), revealed different effects of the chemical modification on B, C and γ hordein. Bands appeared generally sharper and especially γ hordein was better stained with Coomassie Blue. B and C hordein did not alter their mobility after pyridylethylation, but the three γ-type hordein polypeptides (Shewry et al. 1985), most clearly seen in *hor2ca*, migrated slower in SDS-PAGE. γ1 and γ2 shifted in mobility by 2 to 3 kDa and γ3 by 0.7 to 1 kDa (Fig. 3A, B).

Western blots of pyridylethylated and non-pyridylethylated hordein from Carlsberg II and *hor2ca* were carried out in order to investigate possible changes in antigenicity due to the chemical modification of the cysteines. BI, BII and BC antibodies were detected with perodixase-, BX and X with alkaline phosphatase-conjugated secondary antibodies (Fig. 3A, B).

Pyridylethylation had no major effect on the specificity and antigenicity of the five monoclonal antibodies. BI and BII antibodies recognized co-migrating polypeptides, both in pyridylethylated and non-pyridylethylated Carlsberg II hordein. Occasionally, as seen in Fig. 3A, the BI antibody gave a weaker reaction with the higher molecular weight B hordein polypeptides. The reactions of the BC antibody with Carlsberg II and *hor2ca* were identical, with both pyridylethylated γ3 hordein polypeptides identified at a higher position. Varietal differences in BX reaction appear to be caused by a generally weaker γ1 reaction and the recognition of non-mobility shifting B hordein polypeptides in Carlsberg II. X shows a similar behavior with Carlsberg II and *hor2ca* hordein: a broad doublet resolves into a slower moving co-migrating doublet after pyridylethylation.

Specificity of the five monoclonal antibodies towards hordein polypeptides separated by 2D gel electrophoresis

Carlsberg II and *hor2ca* pyridylethylated hordein was resolved by 2D electrophoresis (IEF + SDS-PAGE), followed by Western blotting and immune assay, in order to further characterize the BI, BII, BX, X and BC antibodies with regard to their cross-reactivity and their recognition of individual polypeptides.

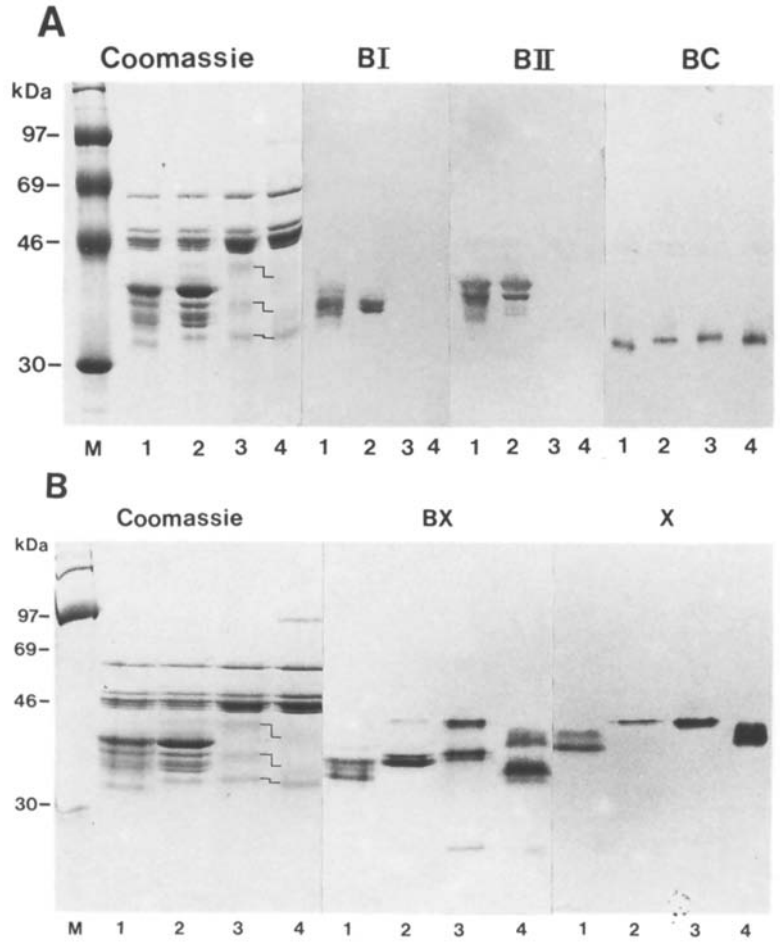

Fig. 3A, B. Comparison of pyridylethylated (PE) and non-pyridylethylated hordein from Carlsberg II and *hor2ca*, separated by SDS-PAGE. Mobility shifts of γ hordein polypeptides induced by pyridylethylation are indicated on the Coomassie Blue-stained gels. The lanes contain hordein from: *1*, Carlsberg II; *2*, Carlsberg II (PE); *3*, *hor2ca* (PE); *4*, *hor2ca*. M, molecular weight markers. Western blots were prepared from replicate gels and assayed with monoclonal antibodies BI, BII and BC, using a peroxidase-conjugated secondary antibody (**A**) and with monoclonal antibodies BX and X, using an alkaline phosphatase-conjugated secondary antibody (**B**)

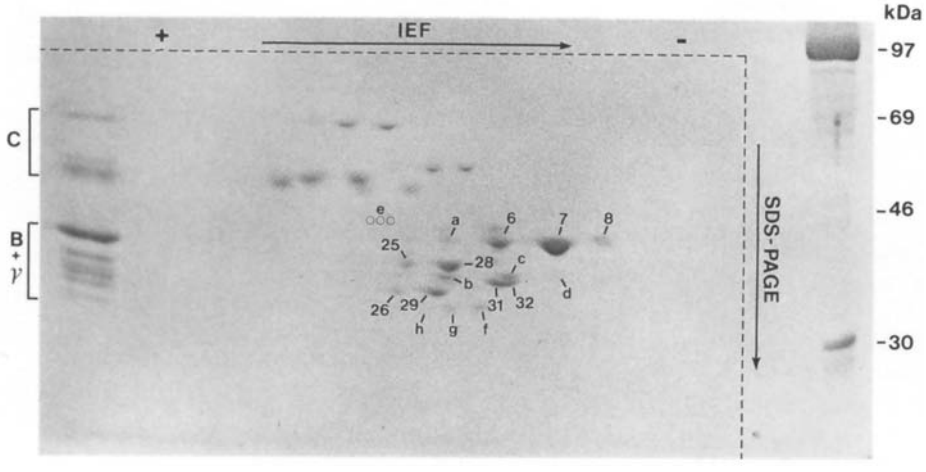

Fig. 4. Pyridylethylated Carlsberg II hordein polypeptides were separated by IEF, SDS-PAGE and Coomassie Blue stained after electro blotting. The B hordein polypeptides were numbered according to Faulks et al. (1981). Additional spots, some only visible after immune assay, were given the letters a to h. Markers run in the second dimension are pyridylethylated Carlsberg II hordein (left) and molecular weight markers (right). The Western blots illustrated in Fig. 5 lie within the area indicated by the dotted lines

Coomassie Blue-stained gels of Carlsberg II (Fig. 4) and *hor2ca* (data not shown) confirmed their common C hordein composition and the absence of B hordein polypeptides in *hor2ca*. The B hordein polypeptides are numbered according to Faulks et al. (1981), while γ hordein and additional spots, some only visible after immune assay, are given the letters a to h (Fig. 4). Immune assays of Western blots of Carlsberg II and *hor2ca*, performed with the five monoclonal antibodies, were aligned with the stained and dried 2D gels, to

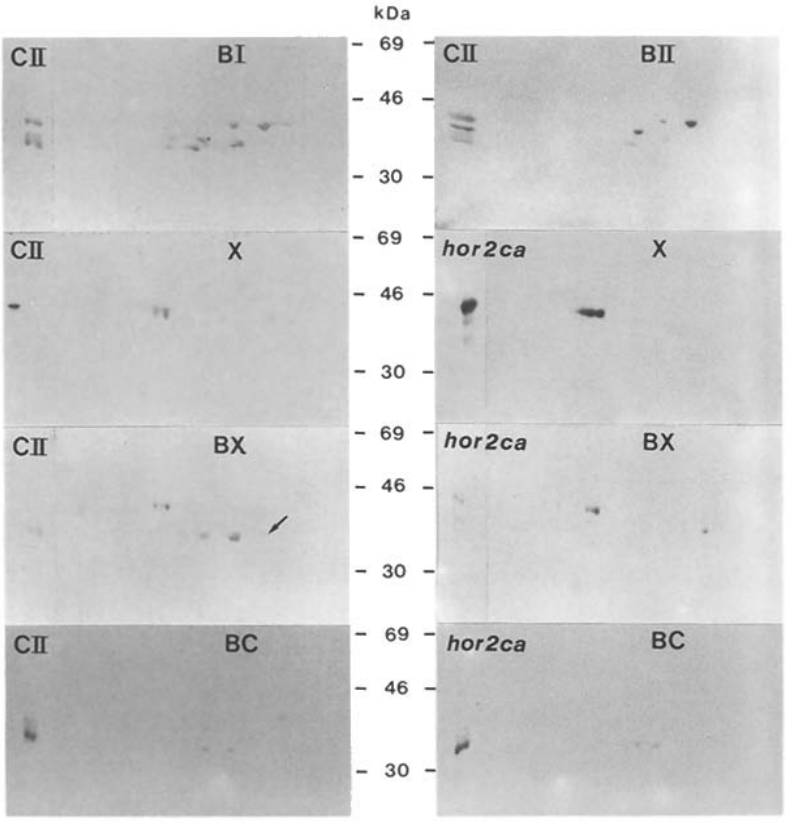

Fig. 5. Western blots of replicate two-dimensional gels of pyridylethylated hordein from Carlsberg II (CII) and *hor2ca*, were incubated with the BI, BII, X, BX and BC antibodies. A reference lane for pyridylethylated hordein separated by SDS-PAGE is present on the left side of each blot

determine the individual polypeptides recognized (Fig. 5).

All B hordein polypeptides were recognized by the BI antibody, whereas BII only recognized polypeptides 6, 7, 28, 29 and 31/32/c. The reaction of BII with polypeptides 29 and 31/32/c was significantly weaker than with BI, indicating that predominantly CNBr cleavage class III polypeptides (Faulks et al. 1981; Shewry et al. 1990) are recognized by the BII antibody. X and BX react with γ1 hordein in identical positions,

Table 1. B and γ hordein polypeptides from Carlsberg II and *hor2ca*, recognized by the monoclonal antibodies BI, BII, X, BX and BC

Polypeptide no.[a]	Molecular weight (kDa)[b]	Hordein group[c]	CNBr cleavage class no.[d]	BI (CII)	BII (CII)	X (CII + *hor2ca*)	BX (CII)	BX (*hor2ca*)	BC (CII)	BC (*hor2ca*)
6	39	B3	III	+ +	+	–	–	–	–	–
7	39	B3	III	+ +	+ +	–	–	–	(+)	–
8	39	B3	III	+	–	–	–	–	–	–
25	37	B3	–	+	–	–	–	–	–	–
26	34	B2	–	+	–	–	–	–	–	–
28	37	B3	III	+ +	+ +	–	–	–	(+)	–
29	34	B2	II	+ +	(+)	–	–	–	–	–
31/32/c	35–36	B2	II	+ +	(+)	–	+ +	–	(+)	–
a	39	B3	–	(+)	–	–	–	–	–	–
b	36	B2	–	+	–	–	?	–	–	–
d	36	γ2	–	–	–	–	(+)	+ +	–	–
e	41	γ1	–	–	–	+ +	+ +)	+ +	–	–
f/g	32	γ3	–	–	–	–	–	–	+	+ +
h	32	γ3	–	–	–	–	–	–	–	+

[a] Faulks et al. (1981)
[b] Deduced from Fig. 3A, lane 2 (Coomassie Blue-stained)
[c] Shewry et al. (1979)
[d] Faulks et al. (1981) and Shewry et al. (1990); CII: Carlsberg II; + +: strong; +: normal; (+): weak; ?: uncertain reaction

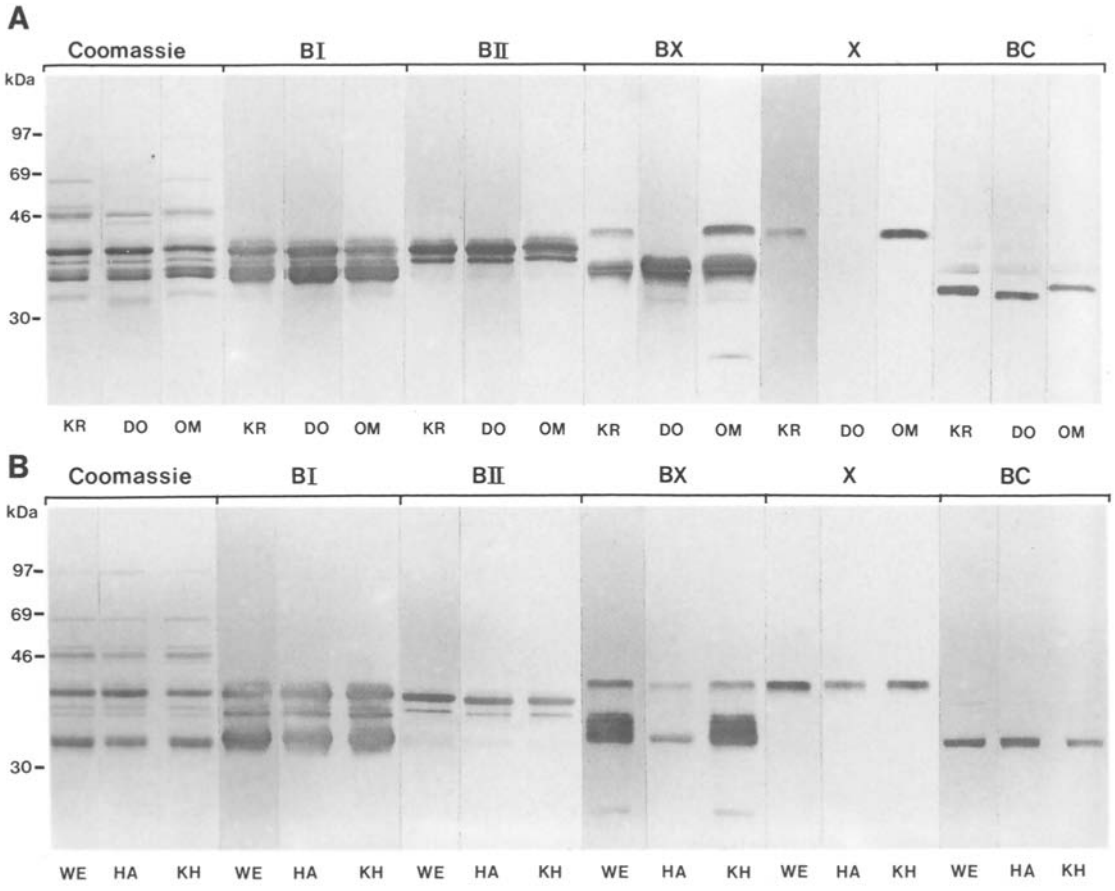

Fig. 6. Hordein extracted from single grains of six barley genotypes (KR, Krasnoufimsky 95; DO, Donetsky 8; OM, Omsky 80; WE, Weihenstephan CP 127422; HA, Haisa; KH, Kharkovsky 70), representing two groups of common B hordein patterns (**A** and **B**), was analyzed by SDS-PAGE and Coomassie Blue staining. The hordein composition was further investigated by immune assays of Western blots of replicate gels with the BI, BII, BX, X and BC antibodies

indicating that γ1 hordein possesses both X and BX epitopes. The BX antibody recognized γ2 hordein and, from the relative strength of the reaction, γ2 hordein appears to be more abundant in *hor2ca* than in Carlsberg II. In addition, BX reacted with the class I/II B hordein polypeptides 31/32/c and b, which are *Hor2*-encoded, since they are not present in *hor2ca*. The BC antibody, which reacted with a single band on one-dimensionally separated Carlsberg II and *hor2ca* hordein, recognized two to three polypeptides, comigrating in the second dimension, and named f, g and h. Interestingly, only γ1 and γ3 hordein polypeptides were found in several isoforms, which may be the products of different genes or the result of post-translational modifications. The fact that γ1 and γ3 hordein isoforms extended over different net pI ranges might indicate that different charge-altering processes occurred. The more abundant B hordein polypeptides in Carlsberg II, namely 7, 28 and 31/32/c, cross-reacted with the BC antibody. The antibody specificity data are summarized in Table 1.

Screening for γ hordein mutants with hordein monoclonal antibodies

Forty barley cultivars with known B hordein composition (starch-gel electrophoresis) were investigated by SDS-PAGE and Western blotting. Coomassie Blue staining revealed several groups with common B hordein patterns (data not shown). These groups were tested with the five monoclonal antibodies. BI, BII and BC antibodies gave a positive reaction with all varieties and the pattern was identical within members of each group. The cultivars from two different B hordein groups, represented by Krasnoufimsky 95, Donetsky 8, Omsky 80 and Weihenstephan CP 127422, Haisa, Kharkovsky 70, were detected with a specific BX and X reaction (Fig. 6A, B).

Donetsky 8 lacked the 41-kDa γ1 hordein band recognized by the X and BX antibodies. BX reacted with a single 33-kDa polypeptide in Haisa, instead of several polypeptides in the range of 33–36 kDa. This suggests that Haisa lacks either γ2 or the B hordein

polypeptides recognized by the BX antibody. The Coomassie Blue-stained polypeptides of Haisa, and their reaction with the BI and BII antibodies, give no indication of a major change in B hordein composition.

Discussion

Apart from a B hordein-like protein, encoded by *Hor4* (*HrdG*) and found in several lines deriving from Elgina (Shewry et al. 1990; Netsvetaev and Sozinov 1984), the majority of the B hordein polypeptides are encoded by the *Hor2* locus (Jensen et al. 1980). Hybrid-selection translation of mRNA encoded by seven different *Hor2* alleles suggested the presence of at least two sub-families of genes within the *Hor2* locus. The polypeptides encoded by these two gene subfamilies correspond to the B hordein CNBr cleavage classes I/II and III (Kreis et al. 1983a). Analysis of the *Hor2* locus in Carlsberg II by pulsed-field gel-electrophoresis (PFGE) revealed that the B hordein genes are contained within two fragments of 160 and 200 kbp, separated by an unknown length of DNA, indicating a physical division of *Hor2* into two subloci (Sørensen 1989). Shewry et al. (1990) reported a rare recombination event within the *Hor2* locus, confirming the division of the B hordein locus into two spatially separated subloci, coding for class I/II and III polypeptides.

The BI and BII antibodies were found to specifically recognize B hordein polypeptides, which is substantiated by the absence of any reaction with hordein from the B hordein-deficient mutant *hor2ca*. BI reacts with all B hordein polypeptides, including the low molecular weight B1 polypeptides which are not present in Carlsberg II. This is most clearly seen in Western blots of barley genotypes (Fig. 6B) with the Bomi *Hor2* band pattern (Fig. 1B), on which the differentiation into B1 (approximately 30–32 kDa), B2 (approximately 33–36 kDa), and B3 (approximately 37–40 kDa) hordein was originally based (Shewry et al. 1979). The BII antibody recognizes predominantly high molecular weight B3 (CNBr cleavage class III) hordein polypeptides. Polypeptide No. 8, also a member of class III, failed to react with the BII antibody. The low abundance of this polypeptide, as indicated by Coomassie Blue staining, may account for this result.

The BX and X antibodies recognize γ hordein polypeptides, present in Carlsberg II and in the mutant *hor2ca*. All γ1 hordein isoforms resolved in 2D gels are recognized by both BX and X antibodies. The disappearance of both reactions in the genotype Donetsky 8 confirms that X and BX epitopes are located on a single polypeptide. The specificity of the BX antibody towards γ2 hordein was confirmed by immune precipitation of in-vitro expressed γ2 hordein. A model is therefore proposed for the location of epitopes on the γ1 and γ2 hordein polypeptides recognized by the BX and X antibodies (Fig. 1C). γ1 and γ2 hordein appear closely related, as they were co-purified and found to share a common N-terminal sequence (Shewry et al. 1985), have the BX epitope in common and perform a similar mobility shift when pyridylethylated. γ1 hordein possesses BX and X epitopes, whereas the smaller γ2 hordein polypeptide carries only the BX epitope. This may have occurred by deletion of an internal or a C-terminal coding sequence containing the X epitope or, alternatively, by insertion of the sequence into the γ2 gene. However, a loss of antigenicity, as a result of a single amino-acid change in the epitope caused by a single point mutation, cannot be excluded. The BX antibody also

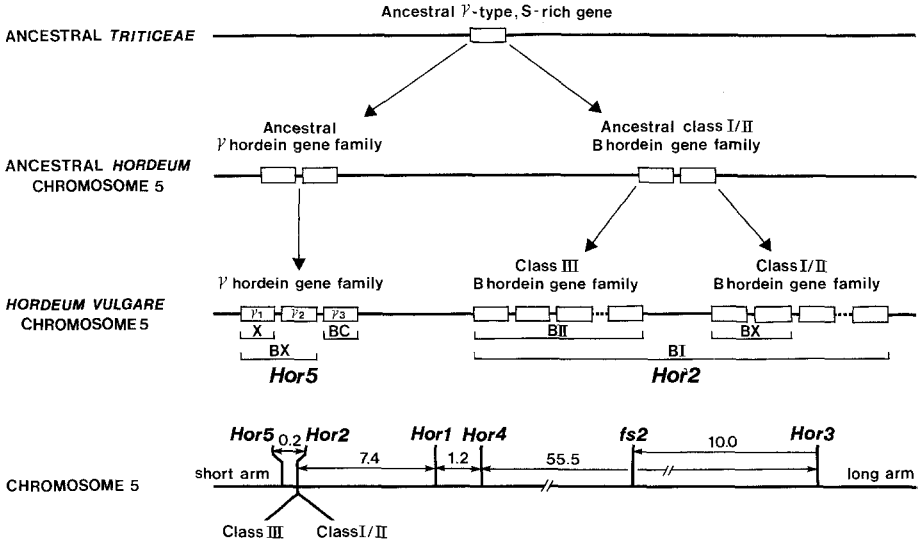

Fig. 7. A model is presented for the evolution of the sulphur-rich gene families in *H. vulgare*. The hordein polypeptides recognized by each monoclonal antibody are indicated beneath the respective genes. A current map of the hordein loci on chromosome 5, taken from Shewry et al. (1990), is given below

reacted with some *Hor2*-encoded B hordein polypeptides of CNBr class I/II in Carlsberg II, and with the same intensity as with γ1 hordein. This reveals a close homology between members of the class I/II B hordein and the γ1 and γ2 hordein polypeptides.

The approximately 28-kDa band reacting on Western blots with BX may be the product of a post-translational cleavage event. It is likely to be derived from a γ1 or γ2 hordein polypeptide, since it is present in *hor2ca* and Nevsky. The absence of a comparable 28-kDa band in in-vitro synthesized polypeptides precipitated by BX is evidence for this view. It is unclear whether the proteolytic event takes place in the tissue (in vivo) or less likely during purification, which was carried out at 60 °C in the presence of 2% β-mercaptoethanol.

The BC antibody recognizes specifically γ3 hordein, but at high antibody concentrations it cross-reacts with all B and C hordein polypeptides. The proline-glutamine repeats of prolamins are known to be highly antigenic (Brett et al. 1990), which suggests that the BC antibody recognizes similar but non-identical epitopes in the repetitive region, common to all hordein polypeptides.

B and γ hordein belong to the sulphur-rich prolamin family. All γ hordein polypeptides are considered to be encoded by the *Hor5* locus, since members of the locus have been mapped within less than 1 cM distal to the *Hor2* locus [0.2 cM: γ2 hordein (Shewry et al. 1990); 0.3 cM: γ3 hordein (Netsvetaev and Sozinov 1982); 0.43 cM: γ1 hordein (Pelger 1991)]. γ3 hordein, although encoded by the same locus, appears less homologous than γ1 and γ2 hordein. γ3 hordein has a different N-terminal amino-acid sequence (Shewry et al. 1985) and is not recognized by antibodies which react with γ1 and γ2 hordein. Furthermore, γ1 and γ2 hordein perform a significantly larger electrophoretic mobility shift after pyridylethylation than does γ3 hordein. The fact that the mobility of B hordein polypeptides is unaffected by pyridylethylation may reflect differences in disulphide bridge formation between the two classes of sulphur-rich prolamins. The formation of intermolecular disulphide bridges between the B hordein polypeptides has been inferred from their failure to migrate in SDS-PAGE under non-reducing conditions, unlike the γ hordein polypeptides in *hor2ca* (Doll 1980). The differences in behaviour of the B and γ hordein polypeptides could be due to the formation of inter-molecular disulphide bridges in B hordein and intra-molecular bridges in γ hordein. This interpretation is supported by an analysis of hordein separated by starch-gel electrophoresis, where B and γ hordein polypeptides are resolved (Pomortsev et al. 1990). When hordein was extracted from seeds under non-reducing conditions (70% ethanol) the recovery of B hordein was particularly low, while C and γ hordein polypeptides were solubilized. The majority of the B hordein polypeptides were only released after inclusion of β-mercaptoethanol during extraction and must, therefore, be stored in an oligomeric form in the grain.

It has been proposed that the prolamins, the α-amylase and trypsin inhibitors from the *Triticeae*, the 2*s* globulins of castor beans and rape, and the chloroform-methanol soluble (CM) proteins of barley and wheat are all members of a super family of proteins, whose genes have evolved from a single ancestral gene (Shewry and Tatham 1990). This gene, which probably encoded a protease inhibitor with a single domain, is thought to have undergone a triplication, giving rise to three homologous domains (A, B and C), remnants of which are conserved within the non-repetitive domains of the prolamin genes. Subsequent insertion of a glutamine–proline encoding sequence, which either previously or subsequently was amplified into a repetitive domain, led to the evolution of the sulphur-rich prolamin genes. Deletion of the A, B and C domains in the course of evolution may account for the almost entirely repetitive domain structure of C hordein, a sulphur-poor prolamin. The presence of sulphur-poor prolamins in wheat (ω-gliadin), rye (ω-secalin) and barley (C hordein) suggests that an ancestral gene evolved in the first grasses. The sequence of the octapeptide repeats in this prolamin group is highly conserved and the consensus motiv PQQPFPQQ can be found in all members. A close homology between S-poor prolamins is further indicated by the recognition of these proteins in wheat, barley and rye using two monoclonal antibodies raised against a total glutenin fraction (Brett et al. 1990). Since the antibodies showed a specific affinity for a synthetic peptide, GQPQQPFPQG, conjugated to BSA, they were probably recognizing the repeat motif common to the monomeric sulphur-poor prolamins.

Two-dimensional electrophoretic resolution of the hordein polypeptides indicates the complexity of the sulphur-poor and sulphur-rich hordein polypeptides. Isolation and characterization of a few C, B and γ hordein polypeptides and their genes has revealed the diversity between these families. In the absence of complete sequences of all the members of the C, B and γ hordein families, monoclonal antibodies provide a valuable tool for determining polypeptide homologies.

Based on our current knowledge of the organization of the *Hor5* and *Hor2* loci and their 15 or more component genes, we have utilized our classification of hordein polypeptides according to common antigenic determinants in order to predict the evolutionary relationship between the sulphur-rich hordein gene families (Fig. 7). It is thought that the ancestral sulphur-rich type prolamin was a γ-type, since this type is present in wheat, barley, rye and temperate

meadow grasses (Shewry and Tatham 1990). Duplication and divergence of this γ-type prolamin gene may have led to the appearance of two distinct ancestral families of γ and B hordein genes. The latter may have been progenitors of the genes encoding the CNBr cleavage class I/II B hordein, since some of their members share common antigenic determinants with the γ1 and γ2 hordein polypeptides.

The ancestral B hordein gene family, by further duplication and divergence, has evolved into two physically separate sub-loci encoding B hordein polypeptides of CNBr cleavage classes I/II and III. Although all B hordein polypeptides share a common epitope recognized by the BI antibody, the BII antibody primarily recognizes polypeptides of CNBr cleavage class III. The divergence of these two subloci may have been a later evolutionary event, since hordein from most wild barley varieties fails to react with the BII antibody (Pelger and von Bothmer 1992).

Less is known about the organization of the *Hor5* locus, but it is apparent that the γ3 hordein gene has diverged significantly from the γ1 and γ2 hordein genes. Whether a physical separation has accompanied this divergence has yet to be ascertained.

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