

A role for $\gamma 3$ hordein in the transport and targeting of prolamin polypeptides to the vacuole of developing barley endosperm

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Summary

Hordein synthesis, transport and deposition was analysed by immunocytochemistry in developing endosperm cells of wild-type (Carlsberg II) and mutant varieties deficient in B hordein (*hor2ca*), $\gamma 1$ hordein (Donetsky), $\gamma 2$ hordein and minor B hordein polypeptides (Haisa), or $\gamma 3$ hordein (Nevsky). In all varieties, hordein polypeptides were detected both in the cytoplasm as globules, ranging in diameter from 50 nm to 1.24 μ m, and in the vacuole as protein bodies. In the cytoplasmic globules B and C hordein polypeptides are assembled as a core and are surrounded by an outer layer of $\gamma 1$ and $\gamma 2$ hordein. The globules apparently fuse several times in the cytoplasm before entering the vacuole. Absence of $\gamma 3$ hordein in the mutant Nevsky leads to a dramatic change in hordein polypeptide targeting, the hordein storage proteins being largely deposited in the lumen of the rough endoplasmic reticulum. $\gamma 3$ Hordein is unique among the sulphur-rich hordein polypeptides, being monomeric and forming only intramolecular disulphide bridges, while the other B and γ hordein polypeptides are aggregated by intermolecular disulphide bridges. Retention of hordein in the rough endoplasmic reticulum in the absence of $\gamma 3$ hordein suggests that $\gamma 3$ hordein may maintain the prolamin storage polypeptides in a transport competent state. The sequence of the mature $\gamma 3$ hordein polypeptide was deduced from a cDNA clone, and compared with $\gamma 2$ hordein. The epitope recognized by the $\gamma 1 + \gamma 2$ hordein-specific BX monoclonal antibody used for immunocytochemistry was mapped to include E¹⁹⁰ and K¹⁹³, by synthesizing overlapping oligopeptides.

Introduction

The germinating barley embryo is dependent on a supply of stored carbohydrate and amino nitrogen, which are deposited in the endosperm during grain development. The mechanism by which seed storage proteins are synthesized, targeted and sequestered for long-term storage in endosperm cells has attracted much interest. Considerable progress has recently been made in understanding targeting and intracellular transport of dicotyledon storage proteins such as sporamin (Matsuoka and Nakamura, 1991) and 11S legumin (Saalbach *et al.*, 1991). There is now convincing evidence for the role of a signal peptide for endoplasmic reticulum (ER) translocation and either a propeptide or internal sorting domains in vacuolar targeting of these storage proteins (Bednarek and Raikhel, 1992). It is generally accepted that the Golgi body plays a central role in intracellular sorting and delivering polypeptides carrying the appropriate targeting signal to the vacuole (Chrispeels, 1991). Glycosylation modification events which accompany Golgi trafficking, provide an invaluable tool for monitoring the progress of transported proteins.

Although many storage protein genes from monocotyledons have been characterized and shown to encode precursor polypeptides with a signal peptide (Kreis and Shewry, 1989), little is known about their targeting and assembly. In particular, it is not known why in some cereals the prolamins are retained in the lumen of the rough endoplasmic reticulum (RER), while in others they are deposited in the vacuole. The vacuolar-targeted prolamins lack cleavable propeptides, although the existence of targeting signals within the mature protein is currently being investigated (Altschuler *et al.*, 1993). The absence of both N- and O-linked glycosylation in most prolamins makes it hard to demonstrate biochemically that intracellular trafficking occurs via the Golgi. The transport of maize prolamins in transgenic tobacco plants has been investigated (Hoffman *et al.*, 1987). Immunocytochemistry of plants transformed with a 15 kDa zein gene showed zein deposition in the vacuolar protein crystalloid of tobacco seeds. Since zeins are exclusively deposited in the lumen of the RER in maize, the relevance of transgenic studies in dicots may be questioned.

The prolamin storage proteins of barley comprise D hordein, homologous to HMW glutenins of wheat, sulphur-poor C hordein and sulphur-rich B and γ hordein (Kreis and Shewry, 1989). The different hordein genes

are coordinately expressed in the developing starchy endosperm tissue (Giese *et al.*, 1983; Sørensen *et al.*, 1989). Hordein precursor polypeptides are co-translationally transported into the lumen of the RER, with simultaneous cleavage of the signal peptide (Cameron-Mills and Madrid, 1989) and are ultimately deposited in the vacuole (Cameron-Mills, 1980; Cameron-Mills and von Wettstein, 1980). Recent progress has been made in assessing the evolutionary relationship between the two subclasses of B hordein and the minor group of γ hordein (Rechinger *et al.*, 1993). In the course of these studies, three barley mutants were identified which lack specific γ hordein polypeptides. Donetsk lacks the 41 kDa $\gamma 1$ hordein, Haisa lacks either the 36 kDa $\gamma 2$ or minor B hordein polypeptides and Nevsky lacks the 32 kDa $\gamma 3$ hordein. In addition, monoclonal antibodies specific for each γ hordein and the major B hordein subclasses were characterized. Antibody X is specific for $\gamma 1$ hordein, antibody BX recognizes $\gamma 1$ and $\gamma 2$ hordein and antibody BC is specific for $\gamma 3$ hordein. Antibody BI recognizes all members of the B hordein family, antibody BII is specific for class III B hordein, while BX recognizes some members of the class I/II B hordein polypeptides.

This paper describes the N-terminal sequencing of three γ hordein polypeptides and a comparison of their primary structure and disulphide bridge formation. The epitope on $\gamma 2$ hordein recognized by the BX monoclonal antibody, is identified with the aid of synthetic oligopeptides. An immunocytochemical study of hordein deposition in wild-type and γ hordein-deficient barley mutants reveals that $\gamma 3$ hordein may play an essential role in intracellular hordein trafficking.

Results

N-terminal sequence analysis of the γ hordein polypeptides

Sequence analysis of pyridylethylated *hor2ca* γ hordein polypeptides, separated by SDS-PAGE and electroblotted on to PVDF (polyvinylidene difluoride) membranes revealed identical N-terminal sequences for $\gamma 1$ and $\gamma 2$ hordein, confirming the results of Shewry *et al.* (1985) with co-purified samples (Figure 1). The N-terminus of $\gamma 3$

$\gamma 1$	?MQVNPSVQV
$\gamma 2$	?MQVNPSVQVQP?QQ
$\gamma 3$	ITTTMQFNPSG
$\gamma 3^*$	ITPPMQFNPSGLELERPQ...

Figure 1. N-terminal amino acid sequences of $\gamma 1$, $\gamma 2$ and $\gamma 3$ hordein.

*Shewry *et al.* (1985).

hordein, however, was found to differ from that of Shewry *et al.* (1985) with three threonines in positions 3–5 instead of triple proline (Figure 1). A second sequence could be clearly differentiated from the main sequence, and was an N-terminal truncated $\gamma 3$ hordein polypeptide, starting at position 8 of the mature protein and containing a proline as the third residue. It is not known if the N-terminal deletion results from proteolytic cleavage *in vivo* or is a purification artefact.

Protein fingerprinting of the γ hordein polypeptides

Limited proteolysis of pyridylethylated γ hordein polypeptides with Glu-C protease, Western blotting and immune reaction with X ($\gamma 1$ specific), BX (reacts with $\gamma 1$ and $\gamma 2$) and BC ($\gamma 3$ specific) antibodies revealed a greater homology between $\gamma 1$ and $\gamma 2$ hordein than between $\gamma 1$ or $\gamma 2$ and $\gamma 3$ hordein (Figure 2). No additional hordein peptides beyond those detected by the antibodies were revealed by silver staining (data not shown). Cleavage was not significantly enhanced when the protease concentration was raised by a factor of 10. However, losses of small peptides may have occurred during extensive washing with 50% MeOH, required for the removal of compounds which interfere with silver staining. BX and X antibodies recognize comparable cleavage products in $\gamma 1$, indicating the relative proximity of the BX and X epitopes. The partial cleavage pattern of $\gamma 2$ hordein was slightly different from $\gamma 1$, with low yields of the smallest recognized fragment, indicating a proteolytic cleavage site in the epitope for BX. Limited proteolysis of $\gamma 3$ hordein failed to resolve pronounced bands, which could be due to a greater number of cleavable residues, compared with $\gamma 1$ and $\gamma 2$ hordein.

Epitope mapping of the BX antibody

Although the sequence of $\gamma 2$ hordein is known (Cameron-Mills and Brandt, 1988), the complex Glu-C protease cleavage pattern of $\gamma 2$ hordein only gave a rough indication of the position of the BX epitope, namely between glutamate 149 and 247, based on the cleavage pattern predicted from the sequence. In order to map the epitope with more precision, 10 oligopeptides of 17–20 residues with an eight amino acid overlap were synthesized (Table 1). After solubilizing the peptides and verifying their correct composition by amino acid analysis, aliquots were dot blotted on to PVDF membrane and incubated with the BX antibody. The immune positive peptide No. 6 was purified by reverse phase HPLC, and the amino acid sequence was determined by N-terminal Edman degradation. The loss of antigenicity of the purified oligopeptide after digestion with trypsin and Glu-C protease (Figure 3)

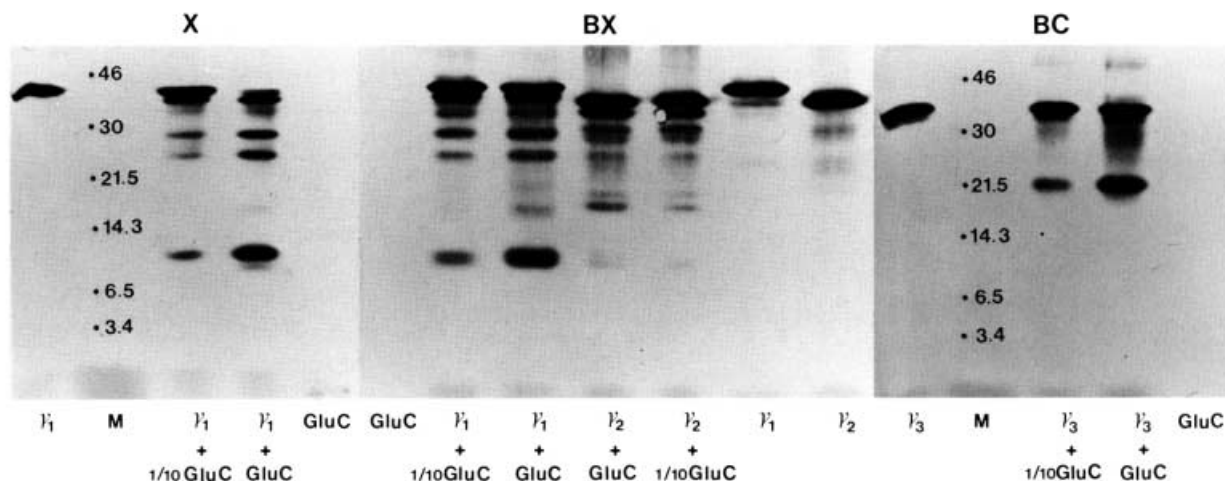


Figure 2. Fingerprinting of the γ_1 , γ_2 and γ_3 hordein polypeptides. Samples were digested with 1 or 0.1 μ g Glu-C protease, subsequently electroblotted on to nitrocellulose and immune assayed with γ_1 specific X, γ_1 and γ_2 specific BX or γ_3 specific BC antibody, and visualized by alkaline phosphatase-conjugated secondary antibodies. M: molecular weight markers (kDa).

Table 1. γ_2 Hordein oligopeptides synthesized by Fmoc chemistry

Peptide no.	aa-aa	Sequence
1	123–142	E Q P Q Q P F F W Q Q P T I Q L Y L Q Q
2	135–154	T I Q L Y L Q Q Q L N P C K E F L L Q Q
3	147–166	C K E F L L Q Q C R P V S L L S Y I W S
4	159–178	S L L S Y I W S K I V Q Q S S C R V M Q
5	171–190	Q S S C R V M Q Q Q C C L Q L A Q I P E
6	183–202	L Q L A Q I P E Q Y K C T A I D S I V H
7	195–214	T A I D S I V H A I F M Q Q G Q R Q G V
8	207–226	Q Q G Q R Q G V Q I V Q Q Q P Q P Q Q V
9	219–238	Q Q P Q P Q Q V G Q C V L V Q G Q G V V
10	231–247	L V Q G Q G V V Q P Q Q L A Q M E

identified glutamate 190 and lysine 193 of the mature protein as essential amino acids of the BX epitope (Figure 4).

Cloning of γ_3 hordein by RNA-PCR

The determined N-terminal sequence of γ_3 hordein (Figure 1) was used to design oligonucleotide primers for the synthesis of a cDNA clone by RNA-PCR (polymerase chain reaction) on *hor2ca* endosperm mRNA. A 959 bp cDNA clone with a poly(A) tail was obtained (Figure 4), whose deduced amino acid sequence beyond the sense primer aligned with the sequence obtained by Shewry *et al.* (1985). The encoded protein has a deduced molecular weight of 33.2 kDa. Analysis of the γ_3 hordein coding

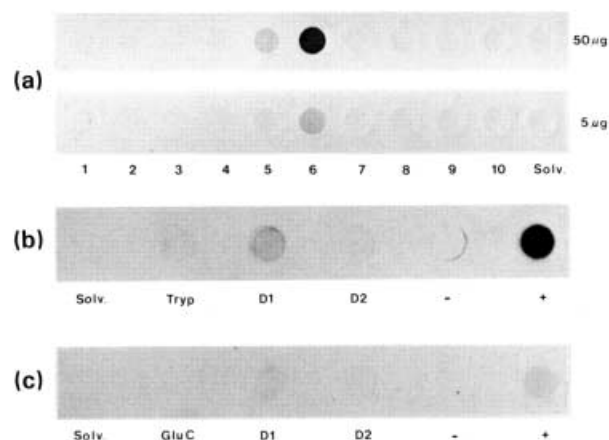


Figure 3. Epitope mapping of the BX antibody by dot blotting of oligopeptides covering mature γ_2 hordein from amino acid 123 to 247 (Table 1), visualized by alkaline phosphatase-conjugated secondary antibodies. (a) Oligopeptides nos 1–10 (50 or 5 μ g), dissolved in 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 2% SDS (w/v) (= Solv.), were dot blotted on to nitrocellulose. (b) Tryptic and (c) Glu-C protease digest of the HPLC-purified immune-positive oligopeptide no. 6. Solv.: Glu-C or trypsin digestion buffer; Tryp: digestion buffer containing 100 μ g trypsin; Glu-C: digestion buffer containing 10 μ g Glu-C protease; -: unblotted; +: oligopeptide positive control (uncleaved); D₁ and D₂: digestion with 10 or 100 μ g trypsin (b) or 1 or 10 μ g Glu-C (c).

sequence reveals two interesting features. First, the nucleotide sequences 213–261 and 249–297 encoding a 16 residue peptide are a direct overlapping repeat. The absence of any substitutions in the repeated sequence suggests that it has arisen by unequal crossing over as a relatively recent evolutionary event. Secondly, the first five residues of the N-terminal sequence of γ_3 hordein are divergent from the γ_2 hordein sequence (Figure 1).

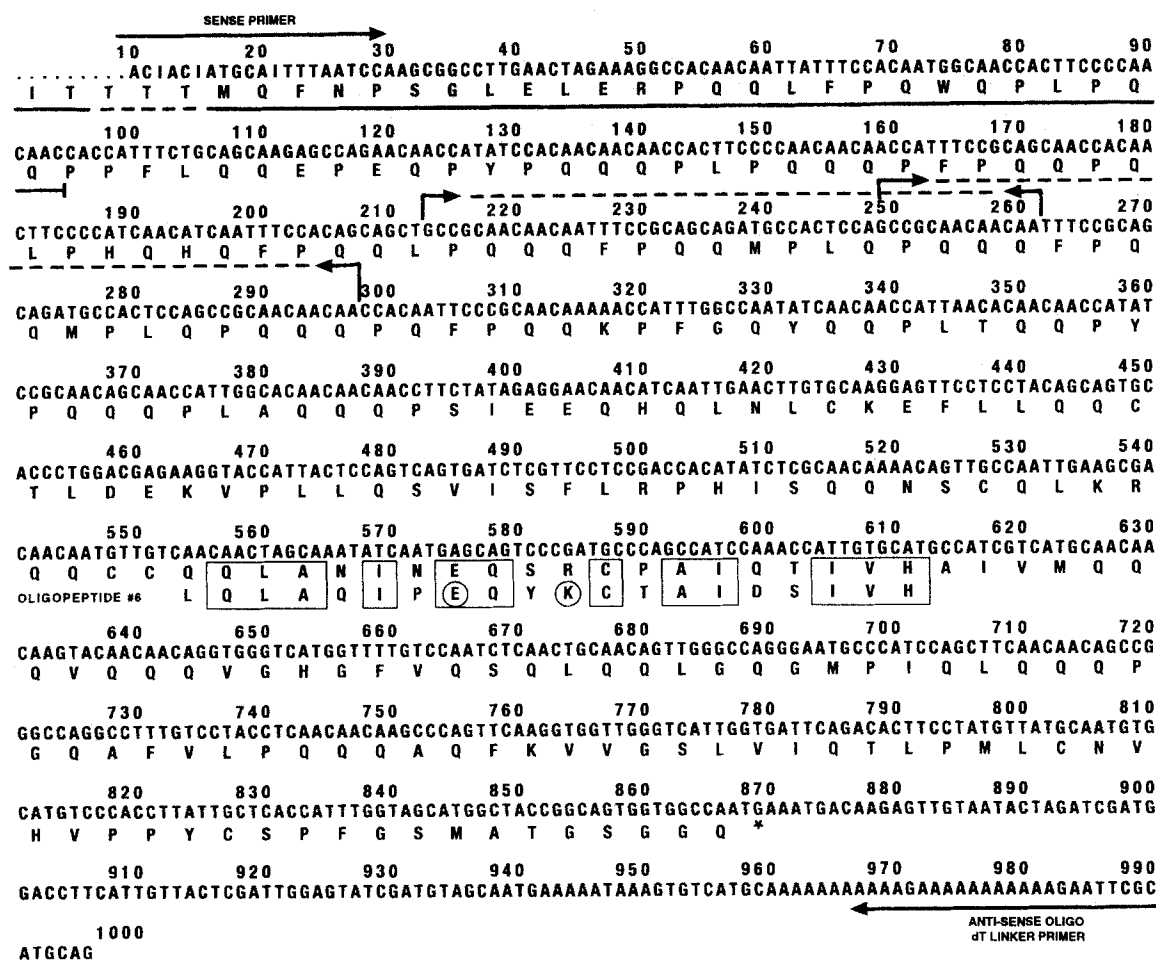


Figure 4. Sequence of a $\gamma 3$ hordein cDNA clone obtained by reverse transcription and PCR of *hor2ca* poly(A)⁺ RNA.

The position of the sense and antisense primers are indicated by arrows. Identity with the determined partial amino acid sequence of Shewry *et al.* (1985) is underlined. A 49 bp, partially overlapping, identical repeat is indicated with dotted lines. The $\gamma 2$ hordein oligopeptide no. 6 (Table 1) recognized by the BX antibody is aligned with $\gamma 3$ hordein, identical residues are boxed and the glutamate 190 and the lysine 193 are circled.

Analysis of inter- and intramolecular disulphide bridges in the S-rich hordein polypeptides in Carlsberg II and mutant *hor2ca*

Extraction and separation of hordein under either reducing or non-reducing conditions revealed that all hordein components are extractable in 55% isopropanol at 60°C, but reduction of intermolecular disulphide bridges was required to resolve individual S-rich hordein polypeptides by SDS-PAGE (Figure 5a). The separation of the γ hordein polypeptides can be seen in the B hordein-deficient mutant *hor2ca*. B hordein polypeptides fail to resolve into single bands and form a smear on the top of the separating gel, when extracted and separated under non-reducing conditions. Reoxidation of the cysteines *in vitro* can be inferred from the loss of resolution of the

S-rich hordein fraction extracted with β -mercaptoethanol and separated under non-reducing conditions (Figure 5a, compare tracks 5 and 7 with 1 and 3). The appearance of faster moving bands indicates the formation of intramolecular disulphide bridges (Figure 5a and b). Carlsberg II hordein extracted and separated under non-reducing condition does not react with the BX antibody (Figure 5a, track 6), indicating that $\gamma 1$ and $\gamma 2$ hordein form intermolecular disulphide bridges *in vivo* and are thus not resolved. The resolution of $\gamma 3$ hordein under both reducing and non-reducing conditions (Figure 5c) indicates the absence of intermolecular disulphide bridges. Its increased mobility in a non-reducing buffer, however, implies the presence of intramolecular disulphide bridges *in vivo* (Figure 5c, compare tracks 6 and 8 with 5 and 7).

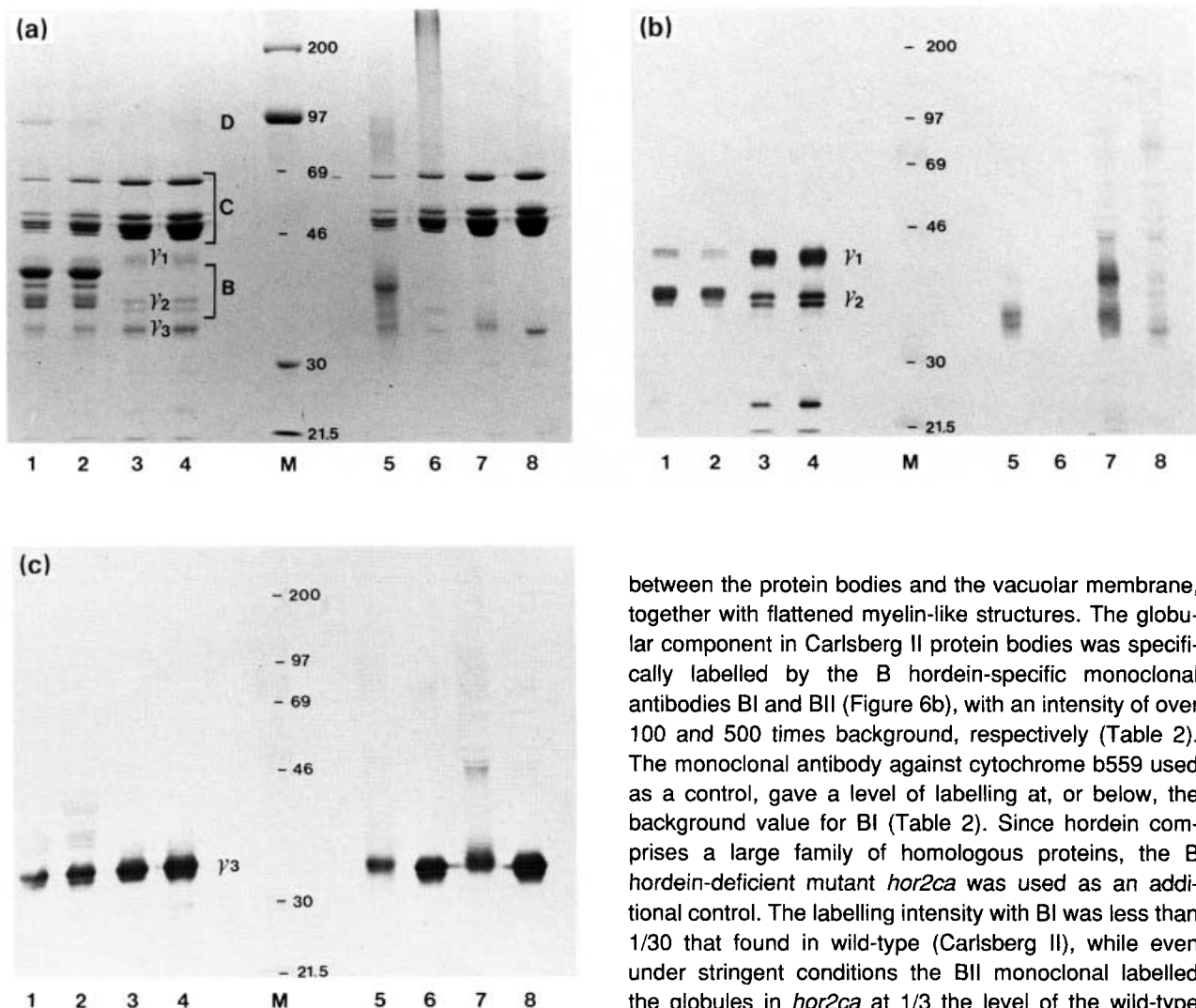


Figure 5. SDS-PAGE and Western blotting of kernels of Carlsberg II and *hor2ca*, extracted and separated under reducing and non-reducing conditions.

(a) Coomassie Blue staining of the blotted gel shown in (b).
 (b) Western blot incubated with the BX antibody.
 (c) Western blot incubated with the BC antibody. 1,2,5 and 6: Carlsberg II; 3,4,7 and 8 *hor2ca*; 1–4: reducing sample buffer; 5–8 non-reducing sample buffer; 1,3,5 and 7: extracted under reducing conditions; 2,4,6 and 8: extracted under non-reducing conditions; M: molecular weight marker (kDa).

Immunocytochemistry of hordein transport

Protein bodies of developing barley endosperm consist of three distinct structures enclosed by a vacuolar membrane. The major component is globular, comprising spherical or ellipsoidal globules of varying sizes, which is associated with a reticulate matrix and small, spherical, electron dense globules (Figure 6a). Numerous small, unilamellar vesicle-like structures were frequently found

between the protein bodies and the vacuolar membrane, together with flattened myelin-like structures. The globular component in Carlsberg II protein bodies was specifically labelled by the B hordein-specific monoclonal antibodies BI and BII (Figure 6b), with an intensity of over 100 and 500 times background, respectively (Table 2). The monoclonal antibody against cytochrome b559 used as a control, gave a level of labelling at, or below, the background value for BI (Table 2). Since hordein comprises a large family of homologous proteins, the B hordein-deficient mutant *hor2ca* was used as an additional control. The labelling intensity with BI was less than 1/30 that found in wild-type (Carlsberg II), while even under stringent conditions the BII monoclonal labelled the globules in *hor2ca* at 1/3 the level of the wild-type (Table 2).

The X and BX monoclonal antibodies, recognizing $\gamma 1$ hordein, and $\gamma 1$, $\gamma 2$ and a small subset of B hordein polypeptides, respectively, also specifically labelled the globules in Carlsberg II (Table 2). A much higher level of labelling was seen in *hor2ca*, consistent with its higher content of γ hordein (Figure 6c). Significant labelling of the reticular matrix was found in *hor2ca*, unlike Carlsberg II, even when the antibodies were further diluted to 1:100 (Table 2).

The BC monoclonal, which recognizes only $\gamma 3$ hordein at high dilution, and additionally B and C hordein at low dilution, primarily labelled the globules of protein bodies in Carlsberg II at dilutions of 1:10 000 and above (Table 2), with an intensity similar to that of BX diluted 1:20. In spite of the stringent conditions used, it was not possible to obtain specific labelling of $\gamma 3$ hordein in Carlsberg II globules without obtaining similar labelling intensities in the $\gamma 3$ hordein-deficient variety Nevsky (Figure 7c, Table 2).

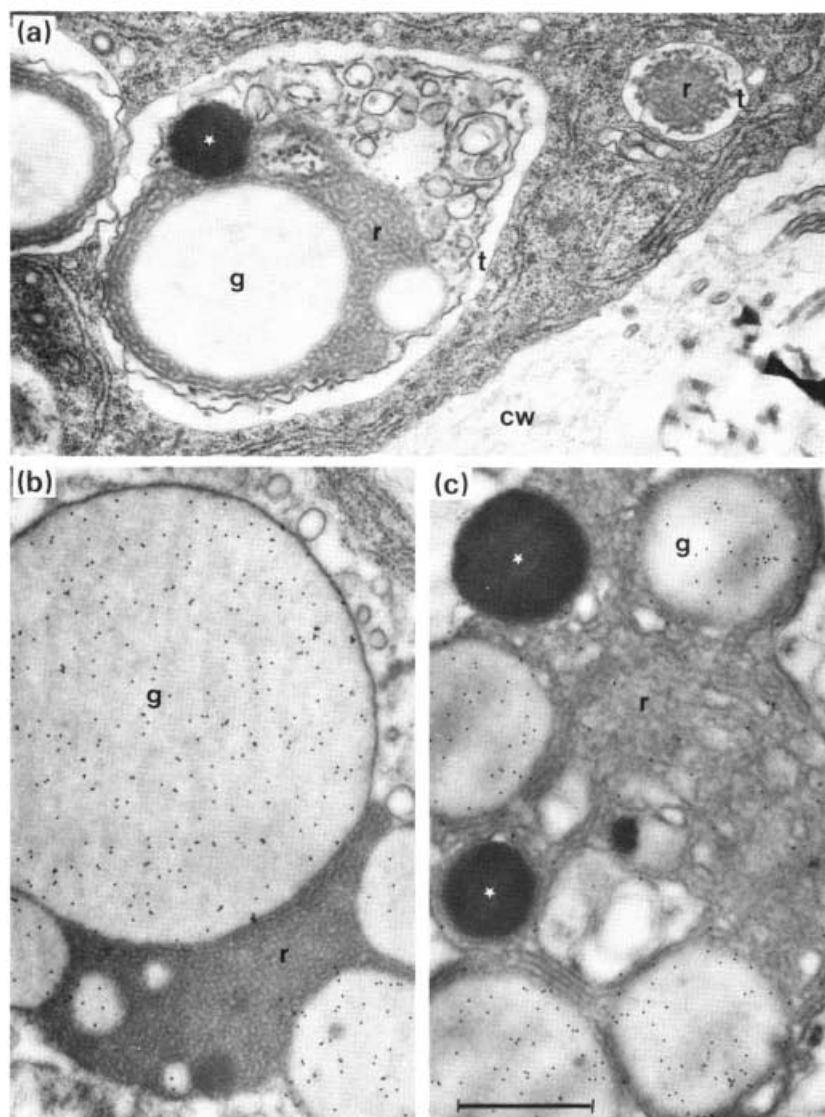


Figure 6. Electron micrographs of protein bodies in the vacuoles of developing barley endosperm

(a). The protein bodies consist of globules (g), electron dense globules (star) and a reticular matrix (r) surrounded by the tonoplast (t). cw: cell wall.

(b) Immunogold labelling of protein bodies of developing endosperm from the wild-type Carlsberg II (in Spurr's resin) using the B1 monoclonal antibody diluted 1:1000, showing localization of B hordeins in the globules (g).

(c) Immunogold labelling of mutant *hor2ca* (in London Resin) using the X monoclonal antibody diluted 1:100, showing localization of $\gamma 1$ hordein primarily in the globules (g) but also in the reticular matrix (r). Bar = 1.0 μm .

In contrast to Carlsberg II, *hor2ca* and other barley varieties examined, Nevsky accumulated large amounts of hordein as globules surrounded by the rough endoplasmic reticulum (RER), which in some instances was seen to be continuous with the rest of the RER (Figure 7a). These globules were generally ellipsoid and approximately the same size as those found within the vacuoles of Carlsberg II and *hor2ca* (Table 3). In cells deeper within the endosperm, almost all of the hordein was deposited within the RER (Figure 7b). In the subaleurone layer hordein was found exclusively as small globules within vacuoles, as in Carlsberg II and *hor2ca*. Between these extremes, cells contained hordein in both the RER and vacuoles.

The sensitivity and specificity of the immunogold labelling enabled the detection of small hordein-contain-

ing globules in the cytoplasm, which are otherwise similar in size, shape and staining behaviour to lipid droplets (Figure 8). In Carlsberg II, the cytoplasmic hordein globules average 0.34 μm in diameter, but range from 80 nm up to 1.24 μm (Table 3)(Figure 8i–k). Similar globules were found in *hor2ca* and Nevsky, where the smallest globules were only 50 nm in diameter (Figure 8h). These are presumptive intermediates in the transport of hordein from their site of synthesis to the vacuole, although no labelling of the RER or Golgi was observed in wild-type barley. The globules did not appear to be surrounded by a membrane, although an irregular outer cortex averaging 30 nm in thickness, was more electron dense. The differences in electron density were reflected in differences in hordein composition, with B and C hordein located preferentially in the interior of the globules, while

Table 2. Differential immunogold labelling of protein body components

Antibody	Structure	Carlsberg II	Mutant
cyt b559 1:100	Globules	0.21 $\pm$ 0.09 (0.05)	Not tested
	Reticulum	0.19 $\pm$ 0.22 (0.11)	
	Rest of cell	0.02 $\pm$ 0.03 (0.02)	
BI 1:1000	Globules	30.2 $\pm$ 2.0 (0.7)	<i>hor2ca</i> 0.89 $\pm$ 0.30 (0.11)
	Reticulum	0.8 $\pm$ 0.4 (0.2)	0.04 $\pm$ 0.09 (0.03)
	Rest of cell	0.25 $\pm$ 0.24 (0.08)	0.03 $\pm$ 0.03 (0.01)
BII 1:3000 ^a	Globules	52.2 $\pm$ 6.3 (2.6)	<i>hor2ca</i> 17.2 $\pm$ 3.3 (1.1)
	Reticulum	1.0 $\pm$ 0.8 (0.3)	0.6 $\pm$ 0.4 (0.1)
	Rest of cell	0.08 $\pm$ 0.02 (0.01)	0.07 $\pm$ 0.04 (0.01)
X 1:20	Globules	4.03 $\pm$ 1.07 (0.4)	<i>hor2ca</i> ^{a,b} 31.8 $\pm$ 11.5 (4.3)
	Reticulum	0.60 $\pm$ 0.40 (0.1)	7.4 $\pm$ 2.2 (0.8)
	Rest of cell	0.14 $\pm$ 0.08 (0.03)	0.14 $\pm$ 0.17 (0.06)
BX 1:20	Globules	12.1 $\pm$ 1.2 (0.5)	<i>hor2ca</i> ^{a,b} 29.6 $\pm$ 2.7 (1.0)
	Reticulum	0.86 $\pm$ 0.51 (0.23)	6.6 $\pm$ 2.5 (0.9)
	Rest of cell	0.14 $\pm$ 0.12 (0.05)	0.16 $\pm$ 0.15 (0.05)
BC 1:10 000 ^a	Globules	11.8 $\pm$ 1.9 (0.6)	Nevsky 15.2 $\pm$ 2.5 (0.8)
	Reticulum	1.1 $\pm$ 0.4 (0.2)	—
	Rest of cell	0.06 $\pm$ 0.05 (0.02)	0.04 $\pm$ 0.03 (0.01)
BC 1:30 000 ^a	Globules	2.11 $\pm$ 0.75 (0.28)	Nevsky 1.72 $\pm$ 0.61 (0.20)
	Reticulum	0.30 $\pm$ 0.38 (0.19)	—
	Rest of cell	0.02 $\pm$ 0.02 (0.01)	0.02 $\pm$ 0.02 (0.01)

Labelling densities are given as particles per $\mu\text{m}^2 \pm$ standard deviation; standard errors are given in parentheses.

^a Indicates stringent incubation conditions with the antibody, due to the presence of 0.2% SDS and 1% Triton X-100.

^b Antibody diluted 1:100.

Table 3. Hordein globule sizes (μm)

Globules	Cytoplasm	ER	Vacuole
Nevsky			
Average	0.23 $\pm$ 0.16	1.21 $\pm$ 0.40	0.75 $\pm$ 0.63
Minimum	0.05 $\times$ 0.05	0.70 $\times$ 0.78	0.16
Maximum	0.51 $\times$ 0.63	3.07 $\times$ 3.07	4.39
Carlsberg II			
Average	0.34 $\pm$ 0.27		1.15 $\pm$ 0.75
Minimum	0.08 $\times$ 0.11		0.13
Maximum	1.09 $\times$ 1.24		4.47
<i>hor2ca</i>			
Average	0.40 $\pm$ 0.15		0.95 $\pm$ 0.61
Minimum	0.15 $\times$ 0.23		0.21
Maximum	0.56 $\times$ 0.80		3.48

γ 1 and γ 2 hordein were concentrated in the outer layer of the globules (Figure 8a–g) (Table 4). The intensity of labelling of the interior of *hor2ca* cytoplasmic globules with X was about the same as found in the vacuolar glob-

ules, whereas the outer cortex was labelled 3 times as intensely (Tables 2 and 4).

Discussion

The identification of barley mutants which fail to synthesize individual γ hordein polypeptides and the characterization of monoclonal antibodies specific for γ 1, γ 2 and γ 3 hordein have enabled us to study this family of hordein polypeptides in greater detail. Homology between the different sulphur-rich hordein polypeptides suggests that γ 3 hordein is quite distantly related to γ 2 and B hordein, while γ 2 hordein shows a close homology to γ prolamins from other cereals, namely γ gliadin and γ secalin (Cameron-Mills and Brandt, 1988). The distinctive nature of γ 3 hordein discussed by Rechinger *et al.* (1993) is further supported by the proteolytic cleavage analysis and epitope mapping of the γ hordein polypeptides. Although γ 1 hordein is $\approx$ 5 kDa larger than γ 2 hordein, their Glu-C

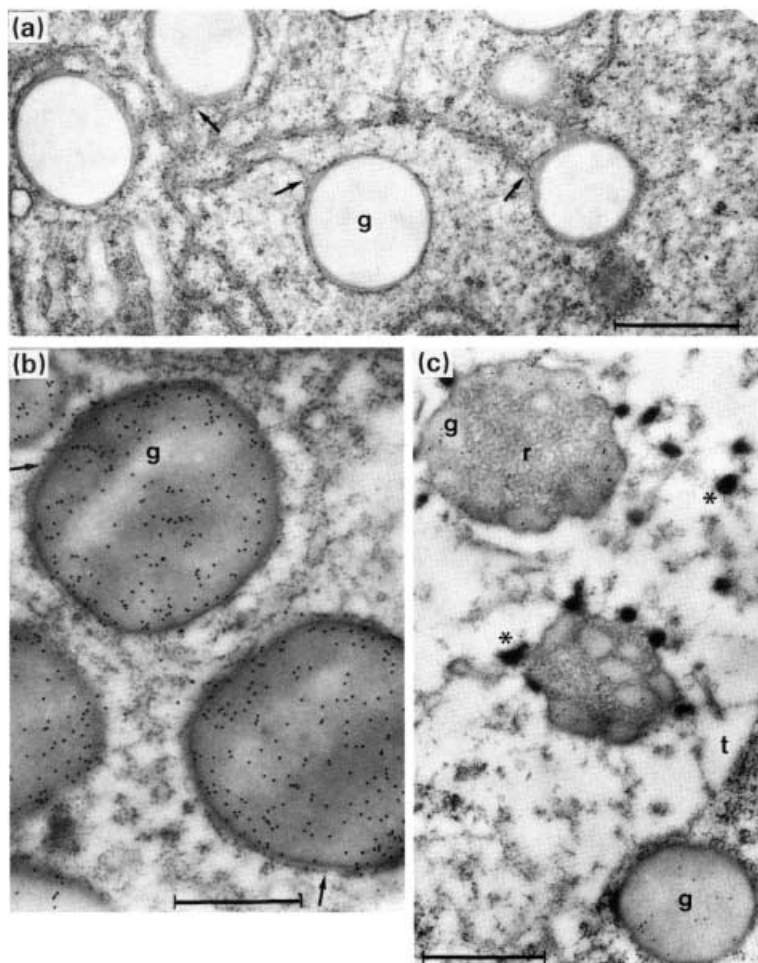


Figure 7. Electron micrograph of developing endosperm of the variety Nevsky, which lacks $\gamma 3$ hordein.

The storage proteins (g) are located in cisternae of the RER, as shown by the presence of ribosomes and continuity with the rest of the RER (arrows). Bar = 1.0 μm .

(b) Immunogold labelling of Nevsky (in London Resin) with the BI monoclonal antibody diluted 1:1000, confirming the presence of B hordeins in the RER of this variety. Arrows indicate ribosomes on the membranes surrounding the hordein globules (g). Bar = 0.5 μm .

(c) Immunogold labelling of Nevsky (in London Resin) with the BC monoclonal antibody diluted 1:10 000. The cross-reaction with B and C hordein makes it impossible to localize the $\gamma 3$ hordeins in Carlsberg II and *hor2ca*. Note the presence of protein bodies in both the RER and the vacuole. The asterisks indicate electron dense material which may be precursors of electron dense globules. Bar = 1.0 μm .

cleavage pattern is related, in contrast to that of $\gamma 3$ hordein. Furthermore, $\gamma 1$ and $\gamma 2$ hordein have identical N-terminal sequences and share a common epitope for the BX antibody, which maps in proximity to the X epitope, unique to $\gamma 1$ hordein. The BX epitope lies in a region of the protein with significant sequence divergence between the different sulphur-rich hordein classes (Cameron-Mills and Brandt, 1988; Shewry and Tatham, 1990).

The native hordein polypeptides, although aggregated, exist as complexes small enough to be solubilized in 55% isopropanol and are equally as efficiently recovered from barley flour under non-reducing as under reducing conditions. The C hordein polypeptides are present as monomers in the extract, while B hordein aggregates are only dissociated after reduction of their intermolecular disulphide bonds. With the aid of the monoclonal antibodies BX and BC, it was discovered that $\gamma 1$ and $\gamma 2$ hordein also form intermolecular disulphide bridges. $\gamma 3$ Hordein was unique among the sulphur-rich hordein polypeptides in

existing as a monomer, with intramolecular disulphide bonds in its native conformation.

The localization of the different hordein polypeptides relative to one another in the protein bodies was investigated by immunocytochemistry with the hordein monoclonal antibodies (Rechinger *et al.*, 1993). In the vacuole of endosperm cells, B and C hordein are exclusively located within the globules of low electron density, together with $\gamma 1$, $\gamma 2$ and possibly $\gamma 3$ hordein polypeptides. In mutant *hor2ca* however, the reticular matrix was also labelled significantly by antibodies recognizing $\gamma 1$ and $\gamma 2$ hordein polypeptides (Table 2).

The existence of hordein globules in the cytoplasm, ranging in size from 50 nm to over 1 μm , was revealed through a combination of excellent ultrastructural preservation and sensitivity of the immunogold labelling. Since labelling was not detected in the RER or Golgi, this implies that the large globules arise by fusion of smaller globules in the cytoplasm. They do not appear to be surrounded by a membrane, but have a non-uniform coating

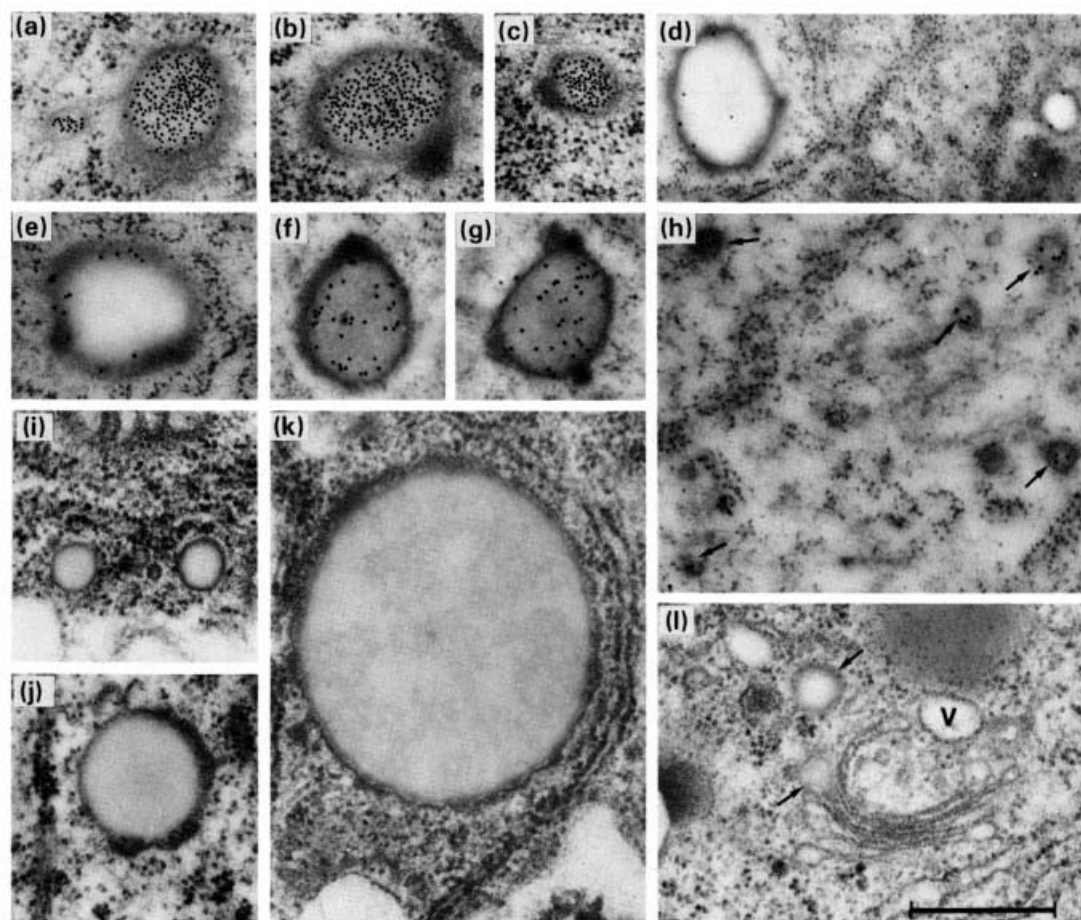


Figure 8. Cytoplasmic hordein globules in developing barley endosperm.

(a–c) Labelling of the interior of cytoplasmic globules of *hor2ca* using the polyclonal antibody recognizing C hordein diluted 1:100 000.

(d) Labelling of the outer layer of cytoplasmic globules of *hor2ca* with the BX monoclonal antibody diluted 1:100.

(e) As for (d), using the X monoclonal antibody diluted 1:100, again showing specific labelling of the outer, darker layer.

(f–h) Labelling of the interior of cytoplasmic globules of Nevsky with the BI monoclonal antibody diluted 1:1000, demonstrating that these intermediates in vacuolar storage protein transport are found in this variety.

(i–k) Cytoplasmic globules from Carlsberg II (OsO₄-fixed, Spurr's resin) showing the wide variation in size and the apparent absence of a surrounding membrane.

(l) Golgi apparatus from the mutant *hor2ca* showing a Golgi vesicle (v) consisting of a membrane surrounding an electron translucent space, and a close association of possible hordein-containing globules (arrows). The involvement of this organelle in hordein transport is not proven.

Bar = 0.5 μ m.

of electron dense material enriched in γ 1 and γ 2 hordein. The localization of hordein polypeptides within the RER of the mutant variety Nevsky confirms, however, that they are co-translationally transported into the RER, as predicted by the presence of a signal peptide and by *in vitro* co-translational transport experiments (Cameron-Mills and Madrid, 1989). It follows that normally the half-life of hordein in the RER must be very short. Since hordein polypeptides are not glycosylated (Cameron-Mills, unpublished results), they may transit through the Golgi even more rapidly. It has been shown that yeast carboxypeptidase moves from the RER to the vacuole with a $t_{1/2}$ of 5 min at 37°C (Winther *et al.*, 1991). Thus, although there is no direct evidence for transport through the Golgi,

this cannot be excluded, at least for a proportion of the hordein. Alternatively, hordein polypeptides may exit directly from the RER into the cytoplasm, as suggested for wheat (Levanony *et al.*, 1992).

The distinctive properties of the hordein polypeptides, namely their solubility in alcohol–water mixtures and their proline/glutamine-rich repeated sequences, has encouraged investigation of their conformation, particularly with regards to their packing and assembly into protein bodies. Circular dichroism and small angle X-ray scattering studies of C and D hordein (Halford *et al.*, 1992; l'Anson *et al.*, 1992; Tatham *et al.*, 1989) predict a rod-like structure based on β -turns. The repetitive N-terminal domains of the B and γ hordein polypeptides may assume a similar

Table 4. Differential labelling of cytoplasmic globules in *hor2ca* with polyclonal antibody recognizing C hordein and the $\gamma 1$ specific monoclonal hordein antibody

Globule	α -hor C 1:100 000	X 1:100
Interior	1580 $\pm$ 490 (131)	37.9 $\pm$ 26.4 (8.3)
Cortex	186 $\pm$ 157 (42)	108.9 $\pm$ 48.4 (15.3)
Interior:cortex	8.50	0.38

Labelling densities are given as particles per $\mu\text{m}^2 \pm$ standard deviation; standard errors are given in parentheses.

conformation. How the cell transports storage proteins with such unique properties has recently been addressed. The secretion of individual wheat prolamin polypeptides was examined by their expression in *Xenopus laevis* oocytes (Simon *et al.*, 1990). γ Gliadin slowly transited through the oocyte secretion pathway while α gliadin largely accumulated in the ER. The transport competence of γ gliadin could be attributed to the C-terminal domain, which is thought to assume a globular conformation with intramolecular disulphide bridges (Altschuler *et al.*, 1993). Expression of the N-terminal repeat domain alone, led to its retention and packaging into protein bodies in the ER. How α gliadin attains transport competence in the wheat endosperm cell remains unanswered, since all the wheat storage prolamins are targeted to the vacuole (Levanony *et al.*, 1992).

If the structural properties of the B and γ hordein polypeptides, and the formation of inter- and intramolecular disulphide bonds among their members have any significance for hordein transport, assembly and packing into protein bodies, these properties should be reflected in altered hordein deposition in barley mutants lacking B or γ hordein polypeptides. Although the structure and localization of hordein polypeptides within the protein bodies in *hor2ca* is slightly modified, the absence of B hordein has no apparent influence on hordein vacuolar targeting. Other mutant varieties examined; Donetsk, lacking $\gamma 1$ hordein and Haisa, lacking $\gamma 2$ hordein or minor B hordein polypeptides, also showed normal hordein deposition. However, the absence of $\gamma 3$ hordein in Nevsky was associated with the accumulation of hordein in ER cisternae, particularly in the deeper endosperm cell layers. The variety Nutans, which has a similar hordein composition to Nevsky, except for the presence of $\gamma 3$ hordein, does not exhibit this phenotype.

In the tropical cereals, maize (Lending and Larkins, 1989), rice (Krishnan *et al.*, 1986) and sorghum (Taylor *et al.*, 1985), the prolamins accumulate within the RER. In pancreatic exocrine cells, zymogens which are normally transported from the RER via the Golgi to secretory granules, can be induced to accumulate in the RER as intracisternal granules by increasing their residence time in the RER, for example by feeding guinea pigs after a

period of starvation, or by using inhibitors such as CoCl_2 or *p*-chlorophenylalanine methyl ester (Tooze *et al.*, 1989). Such protein aggregates exclude immunoglobulin heavy chain binding protein (BiP) and protein disulphide isomerase (PDI), although these ER resident proteins are induced if the zymogens are misfolded. The fact that all the hordein synthesized in the subaleurone cells of Nevsky endosperms is transported to the vacuole suggests that transport competence may be time and concentration dependent. Namely, in those cells where hordein synthesis is slow, the concentration of hordein in the ER lumen is too low for the rapid assembly of transport-incompetent aggregates. It is probable that the prolamins of maize, rice and sorghum are retained in a native conformation in the RER as the result of rapid assembly into aggregates of a size which precludes their further transport to the vacuole. In oats (Lending *et al.*, 1989), wheat (Kim *et al.*, 1988; Levanony *et al.*, 1992; Stenram *et al.*, 1991), rye (Krishnan *et al.*, 1990) and barley a mechanism must have evolved to assemble prolamins in the ER in a transport-competent state. Furthermore, extensive intermolecular disulphide bonding between B hordein polypeptides must be delayed in order to maintain hordein solubility during ER trafficking. This may require the association of transport-incompetent with transport-competent prolamin polypeptides (Altschuler *et al.*, 1993). Positive evidence for the role of $\gamma 3$ hordein in this process requires analysis of the composition and multimerization status of ER-transiting hordein polypeptides. Once the hordein polypeptides have left the ER they are condensed into small globules. The localization of B and C hordein together in the core surrounded by a cortex of $\gamma 1$ and $\gamma 2$ hordein demonstrates that the aggregation of the different hordein polypeptides during intracellular trafficking is not a random process. The roles of the Golgi apparatus and endosomes in this process are the subject of further investigations.

Experimental procedures

Plant material

Barley plants (*Hordeum vulgare* L.), cv. Carlsberg II; B hordein-deficient mutant *hor2ca* (Doll, 1980); Nevsky, lacking $\gamma 2$ hordein; Haisa, lacking $\gamma 1$ hordein; Donetsk, lacking $\gamma 2$ or minor B hordein polypeptides; Krasnoufimsky and Kharkovsky (Rechinger *et al.*, 1993) were grown in pots in a growth chamber with a photoperiod of 16 h light ($250 \mu\text{E m}^{-2} \text{sec}^{-1}$) and 8 h dark at $15^\circ\text{C}/10^\circ\text{C}$.

Monoclonal hordein antibodies

Protein A-purified monoclonal mouse anti-hordein antibodies BI, BII, BX, X and BC (Rechinger *et al.*, 1993; Ullrich *et al.*, 1986)

were stored at -20°C in aliquots, at a protein concentration of 0.2 mg ml^{-1} .

The C hordein polyclonal antibody, raised against a purified gliadin fraction, was kindly provided by Drs H. Skerritt and P. Janes, and its detailed characterization will be the subject of another paper. The monoclonal antibody recognizing the cytochrome b559 of barley thylakoid membranes (Vallon *et al.*, 1987) was used as a control.

Immune assays of Western and dot blots

Immune assays were performed as described by Rechinger *et al.* (1993)

N-terminal sequence analysis of pyridylethylated γ hordein

Pyridylethylated *hor2ca* hordein was prepared and separated by SDS-PAGE as described by Rechinger *et al.* (1993) and electroblotted on to Immobilon-P transfer membranes (Millipore) using a JKA Biotech semi-dry blotting apparatus according to Ploug *et al.* (1989), but omitting MeOH in the transfer buffer (10 mM CAPS [3-(cyclohexylamino)-1-propanesulphonic acid], pH 11 (NaOH)). The position of the γ hordein polypeptides was determined by immune assay with the X, BX and BC antibodies, the corresponding bands were cut out, dried under nitrogen, and stored at -20°C until automated Edman degradation using an Applied Biosystems model 470A gas-phase or model 477A pulsed liquid phase sequencer and an Applied Biosystems model 120 A phenylthiohydantoin-labelled amino acid analyzer.

Fingerprinting of γ hordein polypeptides by limited proteolysis

Pyridylethylated *hor2ca* hordein was separated by 12% SDS-PAGE (Laemmli, 1970) and stained with 0.03% (w/v) Coomassie Brilliant Blue R250, 10% (w/v) trichloroacetic acid (TCA), 40% (v/v) MeOH. γ Hordein polypeptide bands were excised from the gel and stored at -20°C . Sample wells of 16% Tricine-SDS-polyacrylamide gels (Schägger and von Jagow, 1987) were loaded with γ hordein containing gel pieces, equilibrated in cathode buffer and 0.1 or 1 μg sequencing grade Glu-C protease (Boehringer Mannheim) was added. SDS-PAGE was stopped after the formation of a sharp front in the stacking gel. After a digestion period of 2 h at ambient temperature, electrophoresis was continued. Gels were then electroblotted on to 0.45 μm pore size nitrocellulose membranes as described earlier (Rechinger *et al.*, 1993). Silver staining of the gels was performed according to Wray *et al.* (1981).

Peptide synthesis

Oligopeptides were synthesized by multiple column peptide synthesis (Meldal *et al.*, 1993) using Fmoc-(9-fluorenylmethoxycarbonyl) chemistry.

Amino acid analysis

The amino acid composition of the peptides was determined on a LKB, model Alpha Plus, amino acid analyzer after hydrolysis in 6 M HCl at 110°C for 24 h. Glutamine and asparagine were

analysed as glutamate and aspartate, respectively. Cysteine and tryptophan were not determined.

Reverse phase HPLC

Oligopeptides were dissolved in 0.1% (v/v) TFA, 10% (v/v) isopropanol, 10% (v/v) acetonitrile and 1% (v/v) β -mercaptoethanol for cysteine-containing samples. The solubilized oligopeptides were injected and separated on a Waters reverse phase HPLC with a Vydac C₁₈ column at a flow rate of 1 ml min^{-1} with a 50 min linear gradient from 10–100% B (A: 0.1% (v/v) TFA, B: 0.1% (v/v) TFA, 90% (v/v) acetonitrile) followed by 10 min at 100% B. Protein concentration was monitored at 216 and 280 nm.

Epitope mapping of the BX antibody

The oligopeptides *nos* 1–10 (Table 1) were solubilized in 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 2% SDS (w/v) and dot blotted on to 0.45 μm pore size nitrocellulose membranes (Millipore or Schleicher and Schuell) using either Bio-Rad or Schleicher and Schuell equipment, and were subjected to immune reaction with the BX antibody. The immune-positive peptide was purified by reverse phase HPLC and the eluting fractions reprobed with the antibody. The immune-positive fraction was subjected to N-terminal amino acid sequencing.

The HPLC-purified immune-positive fraction was freeze-dried and redissolved in either 1 ml 0.1% (v/v) β -mercaptoethanol, 1 M urea, 0.01% (w/v) SDS, 0.1 M ammonium bicarbonate, pH 7.8, for digestion with 10 or 100 μg trypsin (Sigma) at 37°C , or 1 ml 0.1% (v/v) β -mercaptoethanol, 1 M urea, 0.01% (w/v) SDS, 0.1 M ammonium acetate, pH 4, for digestion with 1 or 10 μg Glu-C protease at ambient temperature. The proteases were added in four aliquots during the incubation time of 20 h.

Construction of a γ 3 hordein cDNA clone by RNA-PCR

The mRNA template for PCR was prepared from spikes of *hor2ca* harvested 20 days after anthesis as previously described (Rechinger *et al.*, 1993). PCR on 0.6 μg poly(A)⁺ RNA from *hor2ca* endosperms was performed with a GeneAmp RNA PCR kit from Perkin Elmer Cetus under the following conditions. The mRNA was annealed and reverse transcribed with an oligo d(T)₁₇ linker primer 5'-CTG CAT GCG AAT TC(T)₁₇ 3' for 5 min at 30°C , and then 20 min at 42°C . The N-terminal sequence from amino acids 4 to 10 were chosen for sense primer construction, incorporating inosine at base positions with three- to fourfold redundancy, and at A/G choices as recommended by Fordham-Skelton *et al.* (1990). The sense primer, with a 7 nt *Eco*RI linker at the 5' end, 5'-GGA ATT CAC IAC IAT GCA ITT T/CAA T/CC C-3' has a calculated T_m of 38.3°C (minus linker) and 53.2°C (plus linker).

PCR amplification was performed on a Perkin Elmer Cetus DNA Thermal Cycler with the following step-cycle programme: Cycle 1: 2 min at 95°C ; Cycle 2: 1 min at 95°C ; 2 min at 37°C (to allow the sense primer to anneal) and 3 min at 72°C ; and Cycles 3–37: 1 min at 95°C , 2 min at 54°C and 3 min at 70°C .

The PCR products were analysed on a 3% (w/v) NuSieve GTG/SeaKem GTG (3:1) agarose gels (FMC Bio Products) and comprised a single DNA fragment of circa 1100 bp. The fragment was recovered and re-amplified with the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer Cetus), using primers

phosphorylated with T_4 polynucleotide kinase (Boehringer Mannheim). The purified 1100 bp fragment was 3' end-filled with T_4 DNA polymerase (Boehringer Mannheim) prior to blunt end ligation into pUC18. The construct was transformed into *Escherichia coli* DH5 α (Hanahan, 1983) and sequenced by the dideoxy chain-termination method of Sanger *et al.* (1977), using Sequenase (United States Biochemicals) and either universal pUC primers or synthetic oligonucleotide primers. All primers were selected according to OLIGO Version 4.0 parameters (National BioSciences, USA) (Rychlik and Rhoads, 1989) and synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer.

Analysis of hordein extracted under reducing and non-reducing conditions

Single, mature kernels were crushed and extracted with 400 μ l of 55% (v/v) isopropanol with or without 2% (v/v) β -mercaptoethanol at 60°C for 2 h with occasional shaking. Following centrifugation for 5 min at 12 000 g, 800 μ l of water was added to 200 μ l of the supernatant, the mixture was divided in two and cooled in liquid nitrogen. After thawing, the hordein precipitates were recovered by centrifugation (5 min, 12 000 g), and the pellet dissolved in 300 μ l SDS-PAGE sample buffer $\pm$ β -mercaptoethanol. The samples were boiled for 2 min and 4 μ l loaded per lane. SDS-PAGE, Western blotting and immune assay with the BX and BC antibody were as described previously (Rechinger *et al.*, 1993).

Electron microscopy

Developing caryopses were harvested between 18 and 22 days after flowering and cut into 0.5 mm transverse sections under freshly prepared 3% (v/v) formaldehyde, 3% (v/v) glutaraldehyde, 10 mM MgCl₂ in 0.08 M PIPES adjusted to pH 8.0 for 1 h at room temperature. Samples were washed with 0.2 M PIPES, pH 6.8 and post-fixed in 0.33% OsO₄ (w/v) in 0.2 M PIPES, pH 6.8 for 1 h (Salema and Brandão, 1973). After two washes in distilled water, samples were chemically dehydrated in two changes of acidified dimethoxypropane (25 μ l 1 N HCl + 50 ml dimethoxypropane) (Dierichs and Dosche, 1982). After washing in 100% ethanol, samples were infiltrated in either London Resin White (medium grade) or Spurr's resin at 50% (1 h), 75% (1 h) and left overnight in 100% resin. Samples were embedded in flat moulds (Spurr's) or in gelatin capsules (London Resin) after two further 1 h incubations in 100% resin, and polymerized for 24 h at 50°C. Thin sections were cut with a diamond knife and picked up on formvar-coated 100 mesh nickel grids. Grids were stained with 5% (w/v) uranyl acetate (30 min at 40°C) and Reynold's lead citrate (30 min at 20°C) before being examined in a Zeiss 902 electron microscope at 80 kV, calibrated with a diffraction grid and catalase crystal lattice spacing (Balzers AG).

Immunocytochemistry

These procedures were carried out in a moist chamber and grids were transferred from 10 μ l drops after excess liquid was removed with filter paper. Thin sections on 100 mesh nickel grids were incubated for 30 min in freshly prepared 5% (w/v) sodium metaperiodate to remove osmium (Craig and Goodchild, 1984). After 5 min washes in water, 0.1 M HCl and water, grids were incubated for 1 h at room temperature in blocking solution to prevent non-specific binding. The blocking solution contained 0.1% (w/v) sodium azide and 1% (w/v) sterile-filtered (0.22 μ m)

skimmed milk in Tris-buffered saline (TBS). The grids were then incubated overnight at 4°C with monoclonal antibodies diluted in TBST (TBS + 0.05% Tween 20) from a 0.2 mg ml⁻¹ stock solution. After washing 5 $\times$ 5 min in TBST, the grids were incubated for 1 h with secondary antibody (goat anti-mouse IgG conjugated to 10 nm colloidal gold, Sigma) diluted 1:40 in TBST. The sections were then washed 2 $\times$ 5 min in TBST, followed by distilled water and then post-stained with uranyl acetate and lead citrate in an Ultrastainer (LKB, Bromma). In some instances, the stringency of the antibody incubation was increased by adding 0.2% (w/v) SDS and 1% (v/v) Triton X-100 to the TBST. Labelling densities were calculated as particles per μ m² using at least seven independent electron micrographs.

Cytochemical controls

The controls consisted of (i) incubation of the sections with gold-labelled secondary antibody alone, (ii) incubation with a monoclonal antibody to chloroplast cytochrome b₅₅₉ diluted 1:100 (Vallon *et al.*, 1987) and (iii) incubation with sections from mutants lacking the storage protein specifically recognized by the monoclonal antibody.

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