Genet. Res., Camb. (1990), **55**, *pp.* 171–176 *With 4 text-figures Printed in Great Britain*

Analysis of a rare recombination event within the multigenic *Hor 2* locus of barley (*Hordeum vulgare* L.)

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(*Received 23 December 1989*)

Summary

A rare recombinant within the multigenic *Hor 2* locus of barley was detected by SDS-PAGE of hordein fractions from F_2 grain from the cross Bomi × P12/3. Analysis of a homozygous F_4 line by 2-D IEF/SDS–PAGE showed that recombination between the class I/II and class III subfamilies of genes had occurred, indicating that they are spatially separate within the *Hor 2* locus. RFLP analysis using a B hordein-related cDNA clone confirmed that recombination had occurred, while similar analysis using a genomic clone related to γ-type hordein (encoded by the *Hor 5/HrdF* locus) indicated the order of the two subfamilies of genes on the short arm of chromosome 5, the class I/II genes being closer to the centromere. The results are consistent with the origin of the B hordein gene family from initial duplication of a single ancestral gene to give two genes which diverged to become the ancestors of the class I/II and class III subfamilies. Subsequent cycles of duplication and divergence have resulted in the present high degree of polymorphism.

1. Introduction

The prolamin storage proteins of cereal seeds are complex polymorphic mixtures of components that are usually encoded by multigene families. In barley, wheat and rye these genes are present as tightly linked clusters that behave as single loci in crosses. Although recombination within these loci is assumed to have contributed to the high degree of variation in the prolamin polypeptide patterns of different genotypes, it is rarely, if ever, observed experimentally.

The *Hor 2* locus, located on the short arm of barley chromosome 5, encodes the B hordein group of prolamins, which account for about 80% of the storage proteins in the mature grain. B hordein is a highly polymorphic mixture; two-dimensional electrophoresis of fractions from eight genotypes showing between 8 and 16 major components, which vary in their proportions, M_rs and pIs (Faulks *et al.* 1981). These polypeptides can be classified into two groups, or subfamilies, on the basis of their cyanogen bromide peptide maps (Faulks *et al.* 1981) and the cross-hybridization behaviour of their cDNAs and mRNAs (Kreis *et al.* 1983*a*). Although 'Southern Blot' analyses of genomic DNA have shown about 20–30 B hordein-related genes per haploid genome (Bunce *et al.* 1986), it has not been possible to determine which genes encode individual proteins. Similarly it is not known whether the two subfamilies of genes are spatially separated within the *Hor 2* locus, or interspersed.

Recombination within the *Hor 2* locus has not been observed, despite the fact that a number of studies of hordein genetics have been made, using a range of genotypes and analytical procedures (Oram *et al.* 1975; Shewry *et al.* 1978, 1980, 1983, 1988; Doll & Brown, 1979; Jensen *et al.* 1980; Blake *et al.* 1982; Shewry & Miflin, 1982). In particular, Shewry *et al.* (1980) used high resolution 1-D SDS–PAGE and 2-D IEF/SDS–PAGE of doubled monoploid lines in order to detect intra-locus recombination, but without success.

In the present paper we report the identification and analysis of a rare recombination event within the *Hor 2* locus. The results indicate that the two major subfamilies of B hordeins are encoded by spatially separated groups of genes within the locus, and linkage with other hordein loci allows us to determine the order of these genes on the chromosome arm.

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2. Methods

(i) *Plant materials and crossing*

Seeds of P12/3 and P12/4 were supplied by Dr V. P. Netsvetaev (All Union Breeding and Genetics Institute, Odessa, USSR). Crosses were made between plants of Bomi and P12/3 grown in the glasshouse at Rothamsted, as described by Shewry *et al.* (1988).

(ii) *Hordein analysis*

Hordein fractions were extracted from milled whole grain and single seeds and separated by 1-D SDS–PAGE and 2-D IEF/SDS–PAGE as described previously (Shewry *et al.* 1988).

(iii) *DNA preparation and transfer*

DNA was isolated from young barley shoots or leaves, essentially using the method of Kreis *et al.* (1983*b*). The DNA was digested according to the manufacturers' instructions (BRL), using 10 μg DNA in a total volume of 50 μl for each digest. The digests were performed for 4 h with sufficient units of enzyme to ensure that there were no partial digests.

Approximately 10μg of each DNA was electrophoresed through a 0·8% agarose gel and transferred to a Hybond N nylon filter (Amersham), essentially as described by Southern (1975).

(iv) *Probe preparation and hybridization*

The DNA probes were radiolabelled with [^{32}P]dATP (Amersham) using the random hexamer method (Feinberg & Vogelstein, 1983). The γ-hordein probe was provided by Dr V. Cameron-Mills (Carlsberg Institute). The pB7 and pCp387 probes were as described by Forde *et al.* (1985*b*). The filters were hybridized with the probe at 65 °C overnight and the non-bound probe removed with SSC, 0·1% SDS, increasing the stringency of the wash from 2 × SSC to 0·1 × SSC. The filters were autoradiographed using Kodak X-Omat film.

For re-use of the filter the probe was removed by washing in 0·4 N-NaOH at 45 °C for 30 min, then neutralized in 0·2 M Tris-HCl pH 7·5; 0·1% SDS; 0·1 × SSC at 45 °C for 30 min.

3. Results and discussion

(i) *Identification and electrophoretic characterization of a recombinant at* Hor 2 *locus*

A number of previously reported studies have established that the two major groups of hordeins, B and C hordein, are encoded by linked loci (*Hor 2* and *Hor 1* respectively) which are located on the short arm of chromosome 5, with *Hor 1* closest to the centromere (Oram *et al.* 1975; Shewry *et al.* 1978, 1980; Doll & Brown, 1979; Jensen *et al.* 1980). A third locus, *HrdF* (*Hor 5*) (Netsvetaev & Sozinov, 1982) is located distally to *Hor 2*, and probably encodes γ-type hordeins (Shewry & Parmar, 1987). In addition a second minor group of hordeins, D hordein, is encoded by a locus (*Hor 3*) located proximally on the long arm of the same chromosome (Shewry *et al.* 1983). The locations of these loci are shown diagrammatically in Fig. 1. The P12/3 and P12/4 lines are derived from the cross Nutans 244 × Elgina (Netsvetaev & Sozinov, 1984) and are isogenic for *Hor 1*, *Hor 2*, *Hor 3* and *Hor 5*, but P12/3 contains an additional B hordein-like protein (arrowed in Fig. 2, track b) which is controlled by a fifth locus, *Hor 4* (*HrdG*), which is located proximally to *Hor 1* (Netsvetaev & Sozinov, 1984; Shewry *et al.* 1988) (see Fig. 1).

Several crosses between P12/3 and other lines were set up to study the linkage relationships of *Hor 4* (Shewry *et al.* 1988). One of these crosses was Bomi × P12/3, and analysis of the hordein patterns of 281 F_2 half seeds by SDS–PAGE revealed one seed with an unusual combination of hordein polypeptides (Fig. 2, track f). The hordein pattern of Bomi is characterized by two major B hordein bands, a diffuse low M_r band called B1 and a high M_r doublet called B3 (Fig. 2, track a). In contrast P12/3 lacks the low M_r B1 band, and has a single major band instead of a doublet in the B3 region (Fig. 2, track b). The F_2 seed

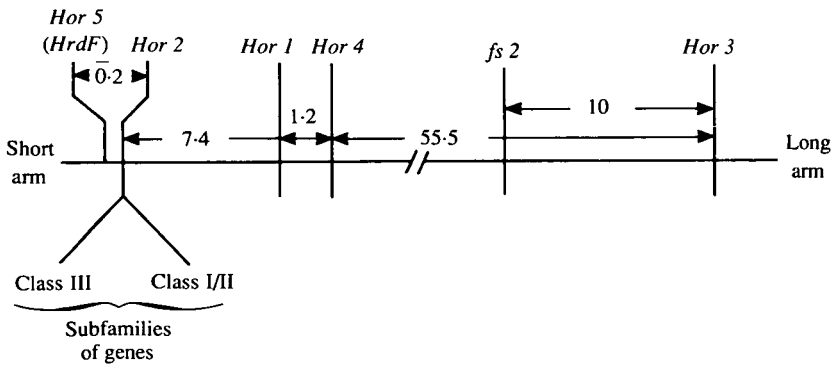

Fig. 1. The locations of structural loci for hordein storage proteins (*Hor 1* to *Hor 5*) on chromosome 5 of barley. The centromere is probably close to *fs2*. The map distances in centimorgans (cM) are based on Jensen (1987).

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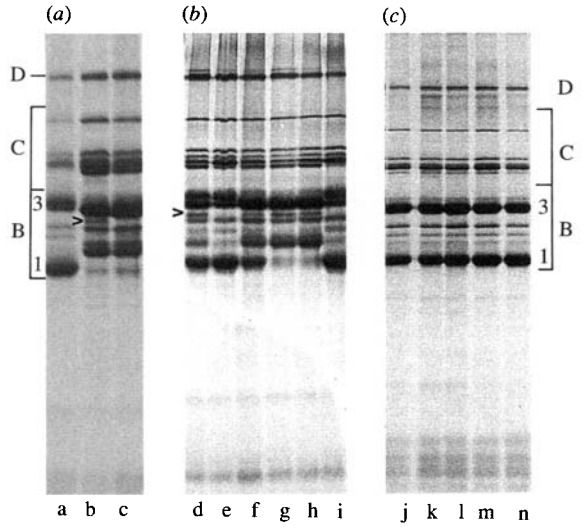

Fig. 2. SDS–PAGE of hordein fractions from single seeds of barley. (*a*) Shows fractions from a, Bomi; b, P12/3 and c, P12/4. The arrow in track b indicates the product of the *Hor 4* locus. (*b*) Shows fractions from F_2 seeds from the cross Bomi × P12/3. The fraction in track f showed an unusual pattern of hordein polypeptides, indicating recombination within the *Hor 2* locus. The arrow in track d indicates the product of the *Hor 4* locus. (*c*) Shows fractions from individual seeds of the homozygous F_4 line 134/31. B, C and D indicate the groups of hordein polypeptides, and 1 and 3 the B1 and B3 bands.

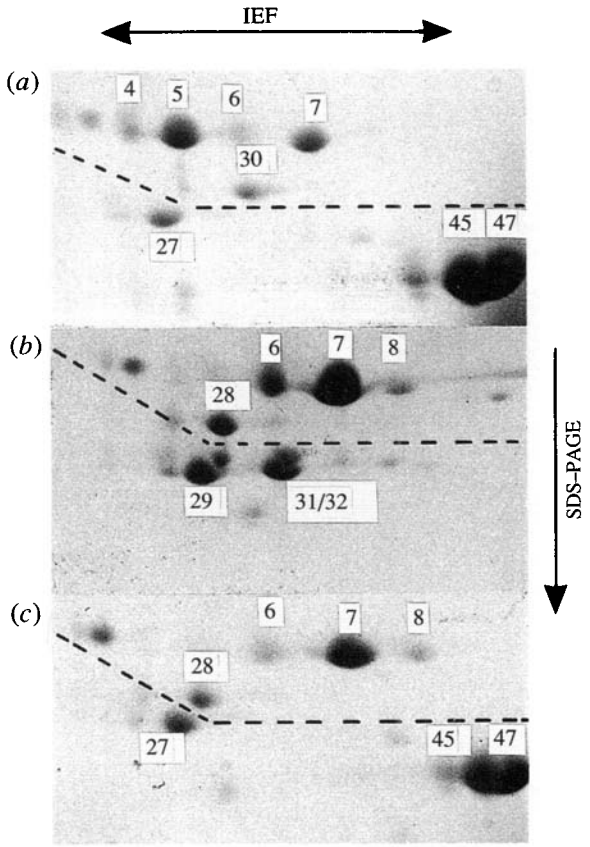

Fig. 3. 2-D IEF/SDS–PAGE analyses of B-hordein fractions from (*a*), Bomi; (*b*), P12/3 and (*c*), recombinant line 134/31. The numbers indicate polypeptides which have been assigned to classes on the basis of their cyanogen bromide peptide maps, those above the broken lines being class III and those below class I/II.

shown in Fig. 2 (track f) was heterozygous, but clearly had the B1 band of Bomi combined with the B3 band of P12/3. This suggested that it resulted from recombination within the *Hor 2* locus.

The embryo half of this seed was grown up and individual seeds were selected in the F_3 generation for homozygosity. This gave a series of F_4 lines that were homozygous for the recombinant B hordein pattern, some of which also had the *Hor 4* gene product. One of the recombinant lines without the *Hor 4* gene product (line 134/31) (Fig. 2*c*) was selected for detailed analysis.

Hordein fractions were extracted from milled grain of Bomi, P12/3 and the recombinant line (134/31), and separated by 2-D IEF/SDS–PAGE (Fig. 3). Bomi (Fig. 3*a*) and P12/3 (Fig. 3*b*) have identical B-hordein patterns to Julia and Carlsberg II respectively which were studied by Faulks *et al.* (1981). It is, therefore, possible to assign the major polypeptides to classes based on their cyanogen bromide peptide maps. Components 4–8, 28 and 30 were shown by Faulks *et al.* (1981) to be class III, and components 27, 31, 32, 45 and 47 to be class I/II. Re-examination of component 29 showed that it was also class I/II, not class III as reported previously (Kreis *et al.* 1983*b*).

In order to determine the parental origin of the B hordein components in the recombinant line the fraction was separated by 2-D IEF/SDS–PAGE, both alone (Fig. 3*c*) and as mixtures with hordein fractions from the two parental lines (not shown). This showed the presence of polypeptides 6–8 and 28 (all class III) from P12/3, and polypeptides 27, 45 and 47 (class I/II) from cv. Bomi. This demonstrates that recombination has occurred between the class I/II and class III sub-families of genes within the *Hor 2* locus, indicating that they are present as spatially separated clusters.

(ii) *Restriction fragment analysis of the* Hor 2 *locus*

Restriction fragment length polymorphism (RFLP) analysis was used to demonstrate conclusively that recombination had occurred within the *Hor 2* locus. Total genomic DNA was prepared from Bomi, P12/3, P12/4 and the *Hor 2* recombinant (134/31), digested with two restriction endonucleases (*Eco*R I and *Hin*d III) and separated on an agarose gel. The fragments were transferred to a nylon membrane and probed with ^{32}P-labelled cDNA. Although the clone selected as a probe (pB7) encodes a B hordein of the class III (B3 hordein) sub-family (Forde *et al.* 1985*b*), previous studies showed that it hybridized to class I/II and class III genes on Southern Blotting (Forde *et al.* 1985*b*; Bunce *et al.* 1986). The patterns of hybridizing fragments are shown in Fig. 4*a*.

The results with Bomi are similar to those reported previously (see Kreis et al. 1984; Bunce *et al.* 1986), with a number of hybridizing fragments, ranging in

size from about 2 to 20 kb. Previously reported copy number reconstructions indicated that the fragments contained between 1 and 10 copies of B hordein genes (see Kreis *et al.* 1984). However, this number must be treated with caution as the intensity of hybridization could be affected by the degree of homology of the gene with the probe as well as the gene copy number.

P12/3 and P12/4 had very similar RFLP patterns, except that the *Hind* III digest of P12/3 contained an extra hybridizing fragment of about 14 kb (arrowed in Fig. 4, track f). This minor difference was reproducible between separately prepared samples of genomic DNA, and may have corresponded to the *Hor 4* gene which encodes a B hordein-related protein (see Shewry *et al.* 1988). Although P12/3 and P12/4 had essentially identical hordein patterns to Carlsberg II (as demonstrated by 2-D analysis) their RFLP patterns differed (compare Figs 3*c* and 4*a* with results in Kreis *et al.* 1983*b*).

The recombinant line (Fig. 4*a*, tracks d, h) showed a clear combination of hybridizing fragments from the two parental lines, fragments derived from Bomi being indicated by arrows. In particular, the *Eco*R I digests showed the presence of an intensely hybridizing fragment of about 2·9 kb which was derived from Bomi (arrowed in Fig. 4*a*, track a). This probably encoded the major B1 hordein polypeptides, since Forde *et al.* (1985*a*) isolated a fragment of this size containing a B1 hordein gene from cv. Sundance, which has an identical RFLP pattern to Bomi (see Bunce *et al.* 1986). These RFLP analyses provide conclusive evidence that recombination had occurred within the *Hor 2* locus, and rule out the possibility that the differences between the B hordein patterns of the parental and recombinant lines arose from post-translational processing events.

(iii) *Restriction fragment analysis of the* Hor 5 (HrdF) *locus*

It was concluded from the electrophoretic and RFLP analyses reported above that the two subfamilies of genes at the *Hor 2* locus were spatially separated. In order to determine the order of these clusters with relation to the centromere, we carried out RFLP analyses of the *Hor 5* (*HrdF*) locus.

Hor 5 (*HrdF*) is located about 0·3 cM distally to *Hor 2* (Netsvetaev & Sozinov, 1982), and appears to encode the γ-type hordeins (Shewry *et al.* 1985; Shewry & Parmar, 1987). Because recombination between *Hor 5* and *Hor 2* is very rare, this locus can be used as a marker to establish the order of genes within *Hor 2*.

A genomic fragment encoding a γ-type hordein (Cameron-Mills & Brandt, 1988) was therefore used to re-probe the genomic DNAs from the four lines

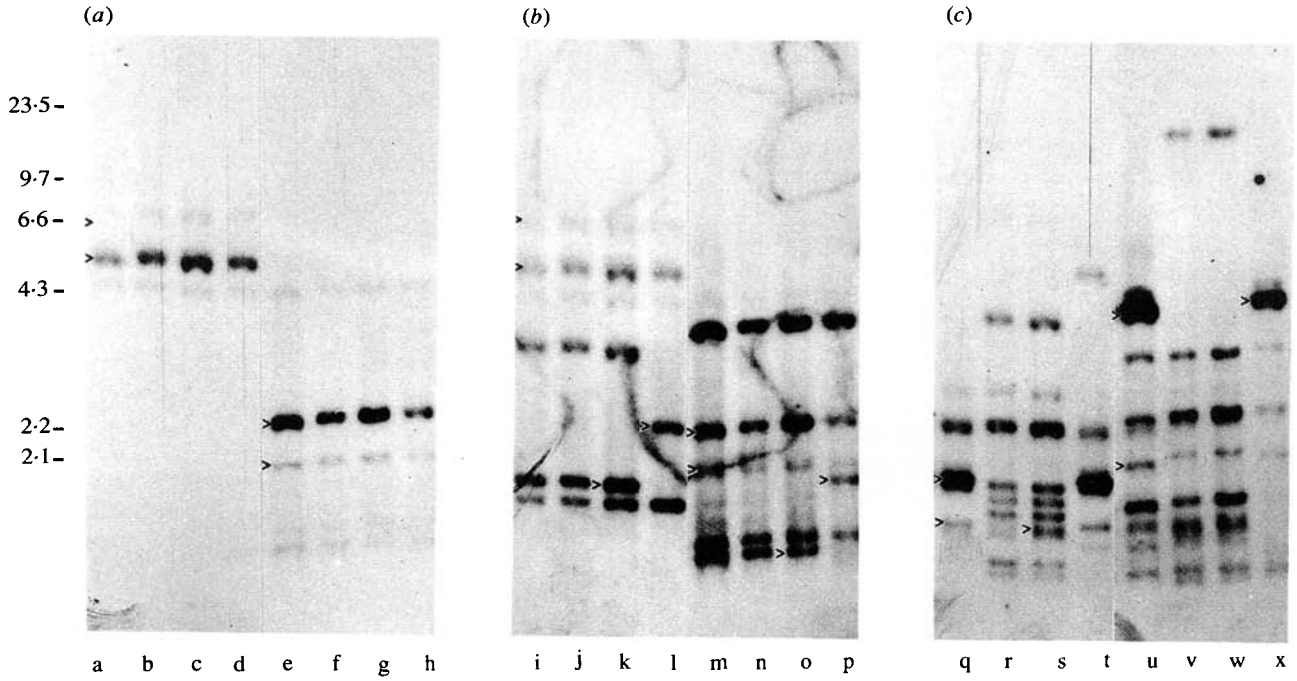

Fig. 4. Hybridization of DNA clones related to (*a*), B hordein (pB7); (*b*), γ-type hordein (λhor -1) and (*c*) C hordein (pCp 387) to *Eco*R I and *Hind* III digests of DNA from Bomi, P12/3, P12/4 and the recombinant line 134/31. Tracks a, e, i, m, q, u are cv. Bomi; b, f, j, n, r, v are P12/3; c, g, k, o, s, w are P12/4, and d, h, l, p, t, x are the recombinant line 134/31. Tracks a–d, i–l and q–t are *Eco*R I digests, and tracks e–h, m–p and u–x are *Hind* III digests. The arrow in track a indicates a fragment of about 2·9 kb which probably encodes B1 hordein and that in track f a fragment which probably corresponds to the *Hor 4* gene. The arrows in tracks d and h indicate fragments present in the recombinant line which are derived from Bomi. The arrows in tracks l, p, t and x indicate fragments which hybridize to the γ-type hordein probe but which probably correspond to C hordein genes. The arrows in tracks i, j, m, n indicate γ-type hordein-related fragments that differ in Bomi from in P12/3 and the recombinant line.

https://doi.org/10.1017/S0016672300025490 Published online by Cambridge University Press

(Bomi, P12/3, P12/4 and 134/31) (Fig. 4*b*). As a control the filter was also probed with a cloned cDNA related to C hordein (Fig. 4*c*). This was necessary because C hordein and γ-type hordeins contain repeated sequences based on the same consensus motif, and some cross-hybridization of cDNAs and genes was expected. This did in fact demonstrate that several fragments that hybridized to the γ-type hordein probe probably corresponded to C hordein genes. These are arrowed in Fig. 4*b*, tracks l and p, and Fig. 4*c*, tracks t and x. All four lines gave identical RFLP patterns with the C hordein probe, which is consistent with their identical C hordein patterns on SDS–PAGE and 2-D IEF/SDS–PAGE (Shewry *et al.* 1988). No cross-hybridization between the probes for γ-type and C hordeins and the genes for B hordein was observed [cf. Fig. 4(*a*) with Fig. 4(*b*, *c*)].

The genomic fragments which hybridized to the γ-type hordein probe but not to the C hordein probe were assumed to correspond to γ-type hordein genes. The pattern of these fragments in Bomi differed from that in P12/3 and P12/4 (see fragments arrowed in Fig. 4*b*, tracks i, j, m, n), with the recombinant line having identical patterns to the P/12 lines. This demonstrates that the *Hor 5* (*HrdF*) allele present in the recombinant line was derived from P12/3 and not from Bomi.

(iv) *General discussion*

The studies described above demonstrate that the recombinant *Hor 2* line has the class III B hordein genes and γ-type hordein genes from P12/3 combined with the class I/II B hordein genes from Bomi. This suggests that the most likely order of genes at the *Hor 2* locus is class I/II – class III – *Hor 5* (see Fig. 1). The opposite order of B-hordein genes could only occur if two recombination events had occurred, between the class I/II and class III sub-families and between *Hor 2* and *Hor 5*. This is highly unlikely in view of the rare occurrences of the two single recombination events.

The demonstration that the class I/II and class III B hordein genes form spatially distinct regions of the *Hor 2* locus indicates that these two sub-families have arisen by the initial duplication of a single ancestral gene, followed by divergence and further duplication events to give two sub-loci. It is not possible to determine the total size of the *Hor 2* locus, but RFLP analyses indicate a copy number of about 20–30 genes per haploid genome (Bunce *et al.* 1986), and Kreis *et al.* (1983*b*) calculated a minimum size of 85 kb based on the total sizes of restriction fragments which were lost in a deletion mutant. The actual size of the locus may be considerably greater, as the restriction fragments may not be contiguous, but interspersed with regions of unrelated DNA sequence. An upper limit to the size may be given by recent studies of Sørensen and colleagues at the Carlsberg Institute (see von Wettstein-Knowles, 1989). They separated large fragments of barley genomic DNA by pulsed field gel electrophoresis and showed that only two fragments of DNA, of 300 and 220 kb, hybridized to a B hordein probe.

Further direct attempts to study the structure and organization of the *Hor 2* locus in more detail may pose problems because the presence of a high proportion (over 90%) of repetitive DNA in cereal genomes (Flavell, 1980) may limit the application of standard procedures of long range mapping such as chromosome walking and jumping. Nevertheless the combination of classical and molecular genetic studies that have been used so far is giving a fascinating picture of how this complex locus has evolved.

We are grateful to Dr V. P. Netsvetaev (Odessa, USSR) for supplying seeds of P12/3 and P12/4, to Dr V. Cameron-Mills (Carlsberg Institute, Copenhagen, Denmark) for the γ-type hordein clone and to Mr P. Sabelli (Rothamsted) for advice on Southern blotting.

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