Theor Appl Genet (1997) 95 : 369–374

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Stability of *Hor1*-specific YAC-clones and physical mapping of *Hor1*-loci in barley

Received: 19 December 1996 / Accepted: 31 January 1997

Abstract The hordeins are the major class of storage proteins in barley and are encoded by multigene families. Two YAC-clones specific for the C-hordein-coding *Hor1*-locus of barley (*Hordeum vulgare* L.) were selected. The clones were constructed with DNA from the cultivars 'Franka' and 'Hockey' and have insert sizes of 330 kb and 350 kb, respectively. Performing partial digestions and hybridizations with vector-specific probes, a restriction analysis was conducted using restriction enzymes with a 8-bp recognition sequence. Both clones cover the complete region of the *Hor1*-locus, but exhibit a different pattern of restriction sites reflecting the polymorphic nature of the locus on the scale of long-range restriction mapping. The maximal extent of the regions homologous to the *Hor1*-specific probe, pBSC5, was 105 kb in the 'Hockey'-derived YAC and 190 kb in the yeast artificial chromosome constructed with 'Franka'-DNA. Furthermore the high degree of instability observed with the *Hor1*-specific YAC-clones is discussed in conjunction with the structure of the *Hor1*-locus.

Key words Yeast artificial chromosomes (YAC) · Barley · C-hordeins · Physical mapping

Communicated by F. Salamini

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Introduction

Hordeins form the major seed storage proteins of the barley grain and are divided into four groups, designed as B-, C-, D- and γ-hordeins (Shewry 1993). The B- and C-hordeins are encoded by linked loci on the short arm of chromosome 5 (1H), designated as *Hor2* and *Hor1* respectively. Depending on the cross examined, there is considerable variation in the genetic distances between the two loci, ranging from 4.6 cM to 22 cM (Pedersen and Linde-Laursen 1995). Both loci have been subject to detailed analysis regarding protein structure and genetics. For the *Hor1*-locus 34 major polypeptides were identified in six different cultivars and a total copy number of 20–30 genes per haploid genome was estimated (Shewry et al. 1985, Bunce et al. 1986). Due to the polymorphic nature of the locus the hordein genes were used for RFLP analysis and variety differentiation (Bunce et al. 1986; Molnar and McKay 1991). The first results about the physical extent of these multicopy loci were obtained by pulsed-field gel electrophoresis (PFGE) experiments. The *Hor2*-locus could be delimited to a maximum size of 360 kb (Sorensen 1989), whereas the *Hor1*-locus was estimated to have a maximum size of 135 kb in both cultivars examined in the survey carried out by Siedler and Graner (1991). During the past few years, the construction of yeast artificial chromosome (YAC) libraries has been used for the analysis of the genomes of various organisms, including barley (Kleine et al. 1993). This technique offers the possibility to clone large fragments of foreign DNA and therefore makes large stretches of DNA accessible to analysis. Kleine et al. (1993) described a YAC-clone for the *Hor1*-locus, but this clone did not cover the complete region of the locus. For further investigation of the genomic structure and organization of this multicopy locus two YAC-clones originating from the DNA of two different cultivars were selected covering the complete genomic region of the *Hor1*-loci in the two cultivars.

Materials and methods

YAC-library and screening

The YAC-library used in this survey was constructed from DNA of the barley cultivars 'Hockey' and 'Franka'. The high-molecular-weight DNA was cloned into the vectors pYAC4 and pYAC-RC (Kleine et al. 1993; Michalek 1994; Kleine and Jung 1996). In the case of pYAC-RC, genomic barley DNA was totally digested with the restriction enzyme *Mlu*I, whereas a *Eco*RI partially digested DNA was cloned into the vector pYAC4.

For screening purposes the library is organized in pooled batches of 384 individual YAC-DNAs. PCR reactions were performed using the *Hor1*-specific primers and the reaction conditions published by Sorokin et al. (1994); 30 ng of YAC-pool DNA was used as template-DNA. Individual clones were identified by colony hybridization. For colony lifts, sterilized nylon membranes (Pall Biodyne B) were placed onto AHC agar plates (Brownstein et al. 1989) and inoculated with a 96- or 384-pin device. After incubation at 30°C for 36 h the membranes were removed and treated as described (Larin et al. 1991). Clones were identified after hybridization of specific probes and confirmed through DNA isolation, PFGE, and subsequent Southern analysis.

Yeast high-molecular-weight DNA preparation

Individual clones were grown for 2–3 days in liquid AHC or SC media (Rose et al. 1990) lacking uracil and tryptophan. The DNA embedded in agarose plugs was essentially prepared as described in Chandrasekharappa et al. (1992). The density of yeast cells in the agarose plugs was adjusted to 1×10^8 cells/ml. In addition to this method which includes Proteinase K, lithium dodecyl sulphate (LDS) was employed for protein solubilization (Southern et al. 1987). Plugs were incubated for 1 h at 37°C in 20 vol of LDS solution (1%LDS, 100 mM EDTA, 10 mM Tris-HCl, pH 8). The solution was replaced and the plugs incubated overnight under the same conditions. For use in enzymatic reactions the plugs produced with Proteinase K treatment were incubated with PMSF (Chandrasekharappa et al. 1992), whereas the plugs produced with the LDS-method were subject to three washing steps, each in 20 vol of TE buffer (10 mM Tris-HCl, EDTA 1 mM, pH 8).

Physical mapping of YAC-clones

For partial digestions one agarose plug was cut into six pieces each with an average volume of approximately 35 µl. These pieces were incubated in 20 vol of the appropriate restriction enzyme buffer at 4°C with gentle shaking overnight. The buffer was replaced by new buffer, restriction enzyme included, and the volume was adjusted to 250 µl. For a series of partial digestions usually 0, 0.05, 0.25, 1.25, 5 and 25 units of enzyme were added. To allow the diffusion of the enzyme into the plugs, the tubes were held on ice for 4 h. Digestion was carried out for 1 h and was stopped by replacing the buffer with stop solution (10 mM Tris-HCl, 20 mM EDTA, pH 7.5). The enzymes used were *Bss*HII, *Cla*I, *Mlu*I, *Not*I, *Sal*I (Pharmacia Biotech, Freiburg) and *Asc*I, *Pac*I and *Pme*I (New England Biolabs, Schwalbach).

Pulsed-field gel Electrophoresis (PFGE) was carried out on a CHEF DR II apparatus (Biorad, München). Agarose gels (1% agarose) were made from PFGE Agarose (USB, Cleveland) and poured into a Biorad casting apparatus or a standard 20 × 20 cm gel casting unit. Unless otherwise noted the DNA was separated at 14°C with a ramped switching time from 20 to 30 s for 22 h at 5 V/cm. After staining with Ethidium bromide (1 µg/ml) for 30 min the gel was photographed and exposed to 60 mJ UV radiation in a UV-oven (GS Gene Linker, Biorad, München). The gels were soaked in 0.4 M NaOH , 0.6 M NaCl for 20 min and DNA was transferred onto a nylon membrane as described (Kleine et al. 1993). Hybridization was performed at 65°C (Jahoor et al. 1991) followed by subsequent washes in 2× and 0.5 × SSC and 0.2% SDS at 63°C for 30 min.

Plasmid probes

The two probes specific for the right and the left end of the YACs are derived from pBR322 DNA by a *Bam*HI/*Pvu*II double digest which generates a 1.7-kb and a 2.6-kb fragment. The 1.7-kb fragment is specific for the right arm and the 2.6-kb fragment is specific for the left arm (Burke et al. 1987). For the hybridization experiments to identify *Hor1*-specific clones, we used the PCR product and the *Hor1*-specific sequence pBSC5, which was tested for *Hor1* specificity by Siedler and Graner (1991). For the mapping experiments we used only pBSC5 as a C-hordein-specific sequence. The probes were labelled by the random hexamer method in the presence of [α-^{32}P]dCTP (Feinberg and Vogelstein 1983).

Results

Using barley DNA as a positive control, PCR reactions with pools of YAC-clones were performed with the *Hor1*-specific primers. The results are shown in Fig. 1 A. The subsequent hybridization of colony filters with pBSC5 revealed the positive clones (Fig. 1 B), which were re-tested via PCR of a single yeast colony. After confirmation of the specificity, high-molecular-weight DNA of the individual clones was isolated for PFGE-analysis, followed by a hybridization experiment using pBSC5. Two specific YAC-clones could be identified (Fig. 2 A). The 350-kb YAC-clone (y370D03) was constructed with partially (*Eco*RI) digested DNA from the barley cultivar 'Hockey' using the vector pYAC4. The second YAC (y103I03), with a size of 330 kb, originated from a total *Mlu*I digest with DNA from the barley cultivar 'Franka' (Fig. 2 B). It was cloned into the vector pYACRC.

Because of the relatively high degree of instability, great care was taken in the growth of the preparative cultures. For DNA preparations the clones were inoculated from the stock culture onto a YPD plate and 25 independent yeast colonies were each picked into 5 ml of AHC medium. For further analysis, a clone which retained its original size (e.g. Fig. 3 A, lane I) was choosen for five preparative cultures (50 ml of AHC media each) in parallel. As an example Fig. 3 shows the results of eight clones (y103I03), each originating from one single colony of the same YPD plate. It is evident that the clones undergo substantial changes during cultivation (Fig. 3 A). In addition to the original clone (lane I), the occurence of smaller clones (lanes 2–8) is apparent. Hybridization with pBSC5 (Fig. 3 B) revealed that the smaller clones still contain *Hor1*-specific sequences, indicating that the different clones are still specific to the applied probe and are not the consequence of cross contamination in the microtiter plates.

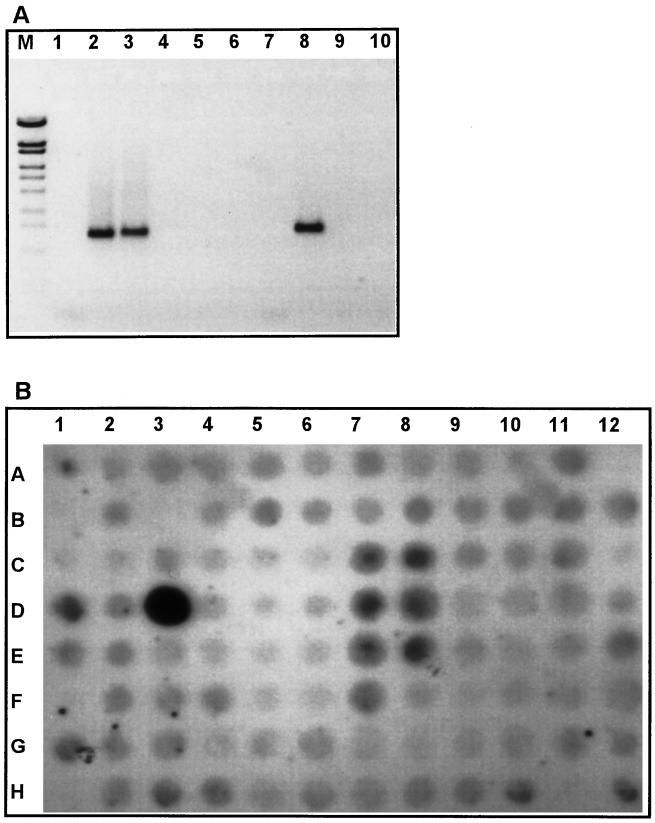

Fig. 1A, B Identification of YACs containing *Hor1*-specific sequences. **A** PCR amplification products with the *Hor1*-specific primers. Water was used as a negative control (*lane 1*), genomic barley DNA as a positive control (*lane 2*), YAC-pools with genomic DNA from 384 individual clones (*lanes 3–8*). The size standard (*lane M*) is λ-DNA/*Eco*130I (MBI Fermentas, St. Leon Rot). **B** Identification of y370D03 after colony hybridization with pBSCs. The exposure time was 3 days

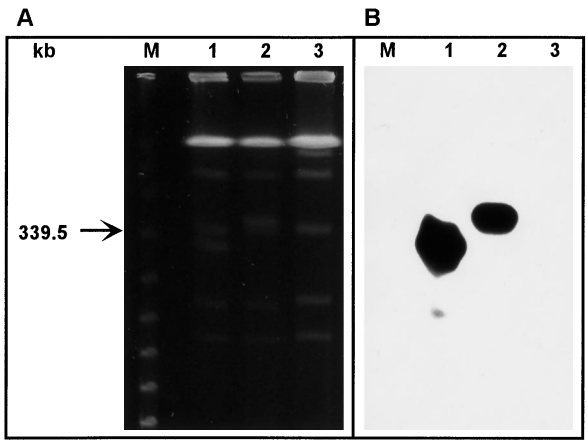

Fig. 2A, B PFGE analysis of the positive YAC-clones. **A** Ethidium bromide-stained PFGE-gel, λ-ladder (*lane M*, New England Biolabs, Schwalbach), y103I03 (*lane 1*), y370D03 (*lane 2*), anonymous YAC (*lane 3*). **B** Autoradiograph after hybridization with pBSC5. The exposure time was 1 day

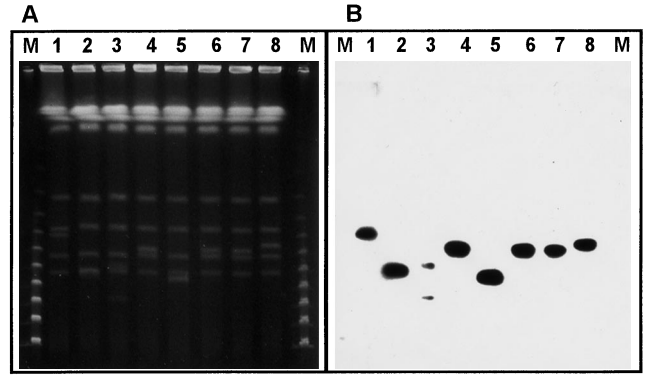

Fig. 3A, B PFGE analysis of separately grown colonies of y103I03. **A** Ethidium bromide-stained PFGE-gel, λ-ladder (*lane M*), y103I03 clones (*lanes 1–8*). **B** Autoradiograph after hybridization with pBSC5. The exposure time was 1 day

The difference in hybridization intensity (e.g. lane 3) can be explained by deletion events including a part of the region where copies of pBSC5 are located. Based on the data of over 50 analytical and preparative cultures for each clone, seven different size classes of smaller YACs, ranging from 230 kb to 320 kb, were observed with y370D03. Instability of y103I03 produced six different kinds of clones, 150–300 kb in size. Moreover, 50% of the y370D03-cultures showed the formation of a 320-kb YAC because of instability; whereas with y103I03 a 290-kb YAC (Fig. 3 A, lanes 4, 6 and 7) was the clone which was observed most frequently (43%). In both cases the other size classes were represented by a similar number of clones. Taking these observations into account, the deletion events seem to be non-randomly distributed within the YAC-clones. In a number of attempts to assess the effects of different media (SC or AHC), instability was not affected by growth in enriched or minimal media.

In an initial RFLP-experiment conducted with the two YAC-clones, as well as with genomic barley DNA and pBSC5 as a probe, a different hybridization pattern between the cultivars was observed (data not shown). In order to reveal the differences in the physical structure of the loci in the two cultivars a PFGE mapping experiment was carried out. The enzymes used for the restriction digests differ not only in the sequence but also in the length of the recognition site. From the enzymes tested only those with a 8-bp recognition site (*Asc*I, *Not*I, *Pac*I, *Pme*I) gave results which were reliably scored. Digests with the 6-bp cutters (*Bss*HII, *Cla*I, *Mlu*I, *Sal*I) resulted in complex hybridization patterns which could not be analysed unambiguously (data not shown). The mapping of the *Not*I sites in the clone y370D03 is shown in Fig. 4 A–C. The vector arm containing the *TRP1* gene is defined as the left arm and the vector arm with the *URA3* gene as the right arm. The complete long-range restriction maps are shown in Fig. 5 and it is apparent that the homology between the two

clones is not perfect. On the 'Hockey'-derived YAC-clone the area containing copies of pBSC5 could be delimited to a maximum size of 105 kb, marked by a *Not*I and a *Asc*I site, and a minimum size of 59 kb, defined by two *Pac*I sites. Within this stretch of DNA only *Pac*I sites could be detected. With the 'Franka'-derived YAC-clone as a substrate, *Asc*I digests generate three fragments with the two fragments on the left showing homology to pBSC5. In combination with the results from *Not*I digestion, the maximal distribution of the pBSC5 copies would be 190 kb. The minimal extent of the locus is determined by a *Asc*I and a *Pac*I restriction site flanking a 78-kb fragment. Similar to the other clone in the area of the pBSC5 copies, several *Pac*I sites could be detected. In contrast, no *Pme*I sites were found, but a cluster of four *Not*I sites is located within 93 kb towards the right end of the clone.

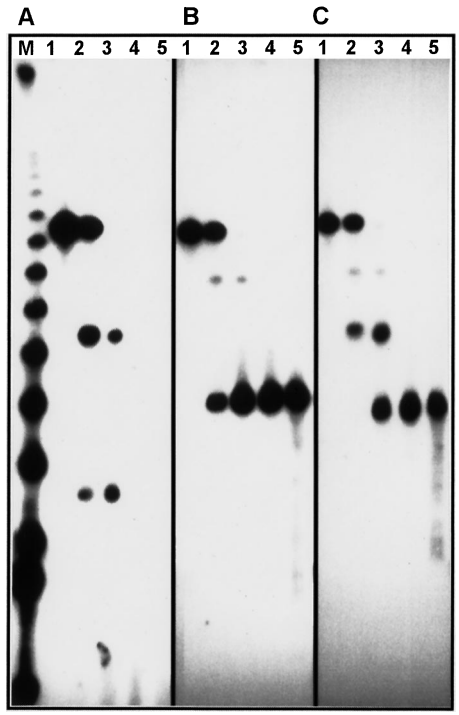

Fig. 4A–C Pulsed-field gel analysis of partial *Not*I digests of y370D03. Partial digestion was performed with 0, 0.05, 0.5, 5, 50 units of enzyme (*lanes 1–5*) and the gel was run with a switching time from 2 to 32 s for 27 h. The size standard (*M*) is λ-ladder + λ-DNA/*Eco*130I. The probes used were right vector arm (**A**), left vector arm (**B**) and pBSC5 (**C**). The *Not*I restriction site near the right vector arm is not visible in this gel system, it was confirmed on a separate gel (data not shown)

Discussion

The *Hor1* -locus has already been the object of detailed studies revealing protein- and RFLP-polymorphisms (Shewry et al. 1985). Siedler and Graner (1991) were able to delimit the physical extent of the locus, but apart from a partially cleaved *Sfi*I site no restriction sites were detected within the region where the *Hor1* gene copies are located. Presumably this is due to the fact that methylation-sensitive restriction enzymes were used and the *Hor1* region is highly methylated in leaf tissue (Sorensen 1992).

The screening of the YAC-library with a *Hor1*-specific primer combination resulted in two YACs with lengths of 330 kb and 360 kb, respectively. Since the source of DNA used for the construction of the YACs was different, we had the opportunity of studying the physical organization of the *Hor1* region in two different cultivars. The extent of the *Hor1*-locus was determined to a maximum size of 135 kb in the cultivars 'Igri' and 'Franka' (Siedler and Graner 1991). We were able to delimit the *Hor1*-region to 105 kb in the 'Hockey'-derived YAC clone. Regarding the position of the *Hor1*-locus in the middle of the clone, it is expected to cover the entire *Hor1*-region. With the second clone, constructed with 'Franka'-DNA, the maximum extent of pBSC5 sequences of the clone was measured as 190 kb. Taking the result of Siedler and Graner (1991) into account, this clone should also cover the complete *Hor1*-region.

For restriction analysis of YAC-clones it has to be borne in mind that *S. cerevisiae* exhibits no methylation activity (Proffitt et al. 1984). In plants CpG, as well as CpXpG, sequences are highly methylated (Gruenbaum et al. 1981) with clusters of non-methylated CpG,

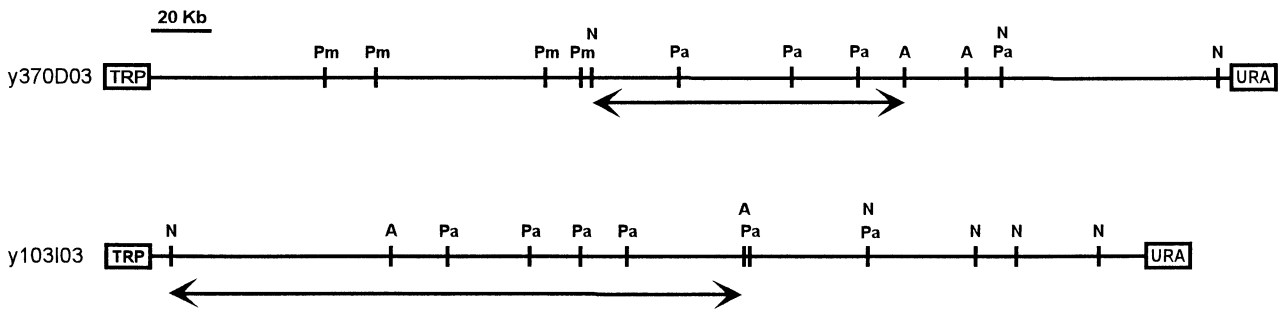

Fig. 5 Restriction map of y370D03 and y103I03. Abbreviations are: *Asc*I (*A*), *Not*I (*N*), *Pac*I (*Pa*), *Pme*I (*Pm*). The *arrows* indicate the regions where copies of the *Hor1*-specific probes were identified

known as CpG islands (Bird 1986), many of which are associated with genes (Antequera and Bird 1987). Therefore, the establishment of co-linearity of restriction patterns between genomic DNA and YAC-DNA is extremely difficult. For the hordein genes it has been hypothesized that methylation is responsible for their inactive state in leaf tissue (Sorensen 1992). For that reason a comparison between the existing physical map, based on genomic barley DNA and the maps derived from the YAC-clones is very difficult, since Siedler and Graner (1991) used DNA from leaf-tissue preparations for map construction. These authors described a CpG island in the vicinity of the *Hor1*-region but no restriction sites within the *Hor1*-region were detected. In the present study only the utilization of restriction endonucleases with a 8-bp recognition sequence produced reliable results. Taking methylation effects into account, the mapping of *Not*I sites in the 'Franka'-derived YAC-clone is consistent with the results from Siedler and Graner (1991). Moreover, with one exception, no CpG-rich restriction sites (*Asc*I, *Not*I) were found within the pBSC5-containing region. Only in the 'Franka'-derived YAC was an *Asc*I site found within the region of *Hor1*-specific sequences, the other GC-rich restriction sites (*Asc*I, *Not*I) are flanking the parts of the clones homologous to pBSC5 and one of these could be part of a CpG island. This is in agreement with results obtained from the human genome, where more than 80% of the restriction sites of these two enzymes are located within CpG islands (Larsen et al. 1992). In contrast, most of the *Pac*I sites (8 of 10) are detected within the region where the genes are distributed. Analysis of six *Hor1*-sequences from the EMBL database (data not shown) revealed that no *Pac*I site is located within these sequences, giving rise to the assumption that *Pac*I sites are a feature of the intergenic regions of the *Hor1*-locus. Whether the use of genomic barley DNA from developing endosperm for the construction of a physical map would give results comparable with YAC restriction mapping depends on the availability of high-molecular-weight DNA from this tissue, and remains a subject for further study.

A remarkable characteristic of the analysed YAC-clones is the high degree of instability. The examination of YACs carrying DNA from organisms other than barley revealed a correlation between sequence organization and the degree of instability. Analysis of YACs originating from the human genome demonstrated, that instability may be correlated with tandemly repeated sequences (Vilageliu and Tyler-Smith 1992) and it has been discussed whether structural stability in YACs reflects stability within the genome (Marshall et al. 1993). Deletions, for example, are favoured by long tandemly repeated elements, which result in the instability of clones derived from the human Y chromosome (Neil et al. 1990). In this case the authors estimated the frequency of rearrangements to be more than one event per generation. In addition, recombination between repeated genes also depends on the length of the sequence, varying from 2×10^{-3} for a 2-kb repeat to 10^{-10} for a 26-bp repeat (Petes and Hill 1988). The structure of the sequence seems to influence this value, too, since the comparison of recombination frequencies between two homologous sequences in a YAC-vector revealed substantial differences (Yasui and Kurosawa 1993). Since rearrangements within the repeated elements at the *Hor1*-locus are assumed to have contributed to the polymorphism of C-hordeins (Shewry et al. 1985), the instability in YAC-clones may be a consequence of this structure. Specific target sites for recombination events would also explain the non-random size of the deleted fragments. Although the exact basis for the instability of clones containing *Hor1*-sequences remains to be determined it seems reasonable to suppose that the *Hor1*-sequence itself, or else associated structures, stimulate mitotic recombination in yeast. This finding might be of particular interest for the employment of YAC clones for the analysis of other complex loci in barley. The complex locus for resistance to powdery mildew (*Mla*), which is located in the vicinity of the *Hor1*-locus, is an especially interesting object for further investigation.

Acknowledgements We thank Katja Grunz for her skilful technical assistance. This work was supported by the BMBF, project No. 0310690.

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