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Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley

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Four minor rDNA loci have been mapped physically to barley (*Hordeum vulgare* L.) chromosomes 1 (7I), 2 (2I), 4 (4I), and 5 (1I) by a two-step *in situ* hybridization procedure including a GAA microsatellite sequence. Reprobing with the microsatellite resulted in a distinct banding pattern, resembling the C-banding pattern, which enabled unequivocal chromosome identification. This study suggests that gene mapping accuracy may be improved by using probes with well-characterized and narrow hybridization sites as cytological markers which are situated close to the gene locus. One of the rDNA loci is located about 54% out on the short arm of chromosome 4 and it has not previously been reported in barley. We have disignated the new locus *Nor-I6*. rDNA loci on homoeologous group 4 chromosomes have not yet been reported in other *Triticeae* species. The origin of these 4 minor rDNA loci is discussed in relation to their equilocal distribution on the chromosomes.

Key words: C-banding, chromosome identification, *Hordeum vulgare*, *in situ* hybridization, microsatellite

Introduction

Fluorescent *in situ* hybridization which provides high resolution is important in human genome projects for the physical mapping of single copy genes (Cherif *et al.* 1989), cosmids (Lichter *et al.* 1990, Cherif *et al.* 1990), and YACs (yeast artificial chromosomes) Selleri *et al.* 1991, Baldini *et al.* 1992) to both metaphase and interphase chromosomes (Lawrence *et al.* 1988, Trask *et al.* 1989). Since this technique was adapted for plant material, knowledge of the physical location of plant genes has increased significantly, and new loci of known genes have been identified (Leitch & Heslop-Harrison 1992, 1993). The increasing number of genes and genetic markers that can be mapped physically to metaphase chromosomes has emphasized the need for new methods to identify and mark individual chromosomes. In barley, the Giemsa C- or N-banding techniques for identification of the single chromosomes are well-developed (e.g., Linde-Laursen 1975, 1981), and it is possible to perform C-banding after *in situ* hybridization (Leitch & Heslop-Harrison 1992). However, it would be desirable to produce a banding pattern simultaneously with *in situ* hybridization. On human chromosomes, this is done by including an *Alu*-sequence in the *in situ* procedure (Baldini & Ward 1991) or by performing R-, G- or Q-banding in the same reaction (Fan *et al.* 1990, Lemieux *et al.* 1992).

This paper describes a two-step *in situ* hybridization procedure in barley which allows the physical localization of a DNA probe to specific chromosomes by including a marker sequence. In addition a sixth chromosome segment carrying the 18S-5.8S-26S rDNA sequence was localized on barley chromosome 4 (4I).

Materials and methods

Plant material

Root tips were obtained from seedlings of *Hordeum vulgare* L. cv. Emir (2-rowed, spring type), cv. Sultan (2-rowed, spring type), cv. Montcalm (6-rowed, spring type), and cv. Igri (2-rowed, winter type). The genome of *Hordeum vulgare* was designated I according to Löve (1984).

Root-tip and chromosome preparation

Meristematic cell divisions were partly synchronized by a hydroxyurea treatment (Doležel *et al.* 1992, Pan *et al.*

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1993). The seeds were germinated on moist filter paper in plastic boxes for 24–32 h at 25°C, and then transferred to filter paper containing a 1 mM hydroxyurea solution for 18h at 25°C in order to stop DNA synthesis. After rinsing in water, the seedlings were incubated on moist filter paper for 6 h, and then pretreated with 0.05% (w/v) colchicine for 2 h before fixation in ethanol-acetic acid (3:1).

Chromosome preparations were made by squashing enzyme-treated root-tips as described by Anamthawat-Jónsson *et al.* (1993). The coverslips were removed by freezing in liquid nitrogen and the slides were dehydrated in 100% ethanol and air-dried. The slides were stored at room temperature for some days or desiccated at −20°C for longer periods.

DNA probes and hybridization mixtures

The wheat ribosomal clone pTA71 (Gerlach & Bedbrook 1979) containing the 18S-5.8S-26S rRNA genes and the nontranscribed spacer sequence was labelled with biotin-14-dATP by nick translation (Gibco Bethesda Research Laboratories, MD, USA). The probe was mixed to a final concentration of 2.5 μg/ml in a hybridization solution (Leitch & Heslop-Harrison 1992) containing 500 μg/ml salmon sperm DNA.

Based on the wheat and barley microsatellite sequence described by Dennis *et al.* (1980), a single-stranded oligonucleotide with the sequence $(GAA)_7$ was synthesized on an automated DNA synthesizer (Applied Biosystem). The 21-mer was end-labelled with biotin-14-dATP by terminal deoxynucleotidyl transferase (Gibco BRL, MD, USA). The labelled oligonucleotide was mixed to a final concentration of 1 μg/ml in the hybridization mixture, as described for the ribosomal probe, except that the formamide concentration was lowered to 15%.

Hybridization and detection protocol

The slides were pretreated in 1 μg/ml DNase-free RNase in 2 × SSC for 1 h at 37°C and washed once in 2 × SSC. They were subsequently incubated in 0.2 μg/ml proteinase K for 8 min at 37°C, rinsed in PBS, dehydrated in ethanol, and air-dried.

The slides were denatured in 0.2 N HCl at 37°C for 10 min, rinsed in ice-cold water, dehydrated in a graded ethanol series at −20°C, and air-dried. The probe mixture was denatured at 95-100°C for 5 min and chilled on ice before 15 μl was loaded onto each slide. The slides were covered with coverslips, sealed with rubber cement, and incubated at 37°C overnight.

After hybridization, the coverslips were carefully removed and the slides washed in 2 × SSC at 37°C for 10 min. Slides were then washed for 10 min in 0.2 × SSC at 60°C for slides with the nick translated probe, and at 37°C for slides with the oligonucleotide probe. The stringent wash should remove hybridized pTA71 sequences with less than about 85% homology (Meinkoth & Wahl 1984) and for the oligonucleotide probe we have calculated that the stringent wash will ensure about 80-85% probe and target similarity. After a further wash in 2 × SSC at 37°C, the slides were transferred into the detection buffer (4 × SSC, 0.2% Tween 20).

Hybridized probes were detected using the biotin-avidin based fluorescence system described by Pinkel *et al.* (1986). We used 5 μg/ml FITC-avidin DCS (Vector) and one amplification with 5 μg/ml biotinylated anti-avidin D (Vector), following the procotol of Lawrence *et al.* (1988). After the final washing, the slides were briefly dehydrated in 70% and 100% ethanol, dried and mounted in an anti-fade solution of 90% glycerol and 10% PBS, pH 8.6, containing 0.005 M *p*-phenylenediamine (Krenik *et al.* 1989), 0.4 μg/ml diamidinophenylindole (DAPI), and 1 μg/ml propidium iodide.

Reprobing

After examination and photography of metaphases which had hybridized with the ribosomal probe, the preparations were reprobed by the method of Heslop-Harrison *et al.* (1992). The coverslips were taken off and the slides were washed three times in detection buffer for 2 h and dehydrated through an ethanol series. Then the hybridization procedure was repeated from the denaturation step using the microsatellite probe.

Microscopy and photography

The slides were examined with a Zeiss Photomicroscope III equipped with a 100 × Neofluar objective and filter sets for DAPI (487702), and for propidium iodide and fluorescein isothiocyanate (FITC) (487709). Photographs were taken on a Kodak Ektachrome P800/1600 professional film for colour slides.

Measurements and calculations of chromosomal distances

Chromosomal distances were estimated on the basis of slide projections to a magnification of about 26,000 times the somatic metaphases. Only isolated chromosomes, which were identified by their banding patterns obtained using the GAA microsatellite probe, were included in the measurements. Further, only those hybridization signals which were visible as double spots were recorded.

The fraction lengths (FLs) (%) of the rDNA hybridization sites were calculated as the distance from the centromere to the hybridization signal relative to the

total length of the chromosome arm. The relative distances from the sites to the positions of the closest GAA-bands (GAA hybridization sites) were also calculated. This expression gives the distance between the hybridization signal and the GAA-band relative to the total arm length. Standard deviations were estimated for all calculations.

Results

In situ *hybridization with the 18S-5.8S-26S ribosomal DNA probe*

The ribosomal DNA probe identified the four major rDNA sites at the nucleolar organizer regions (NORs) of barley chromosomes 6 (6I) and 7 (5I), two rather strong hybridization sites on the two copies of chromosome 5 (1I), and six weaker sites seen as double signals on three other chromosome pairs (Figure 1A). These twelve hybridization sites were found in all four cultivars examined.

In situ *hybridization with the microsatellite sequence*

Reprobing with the GAA microsatellite sequence resulted in a distinct banding pattern on the chromosomes (Figure 1B). In each cultivar it was similar to that obtained by C-banding (Linde-Laursen 1975) except that the terminal bands and the bands at the nucleolar

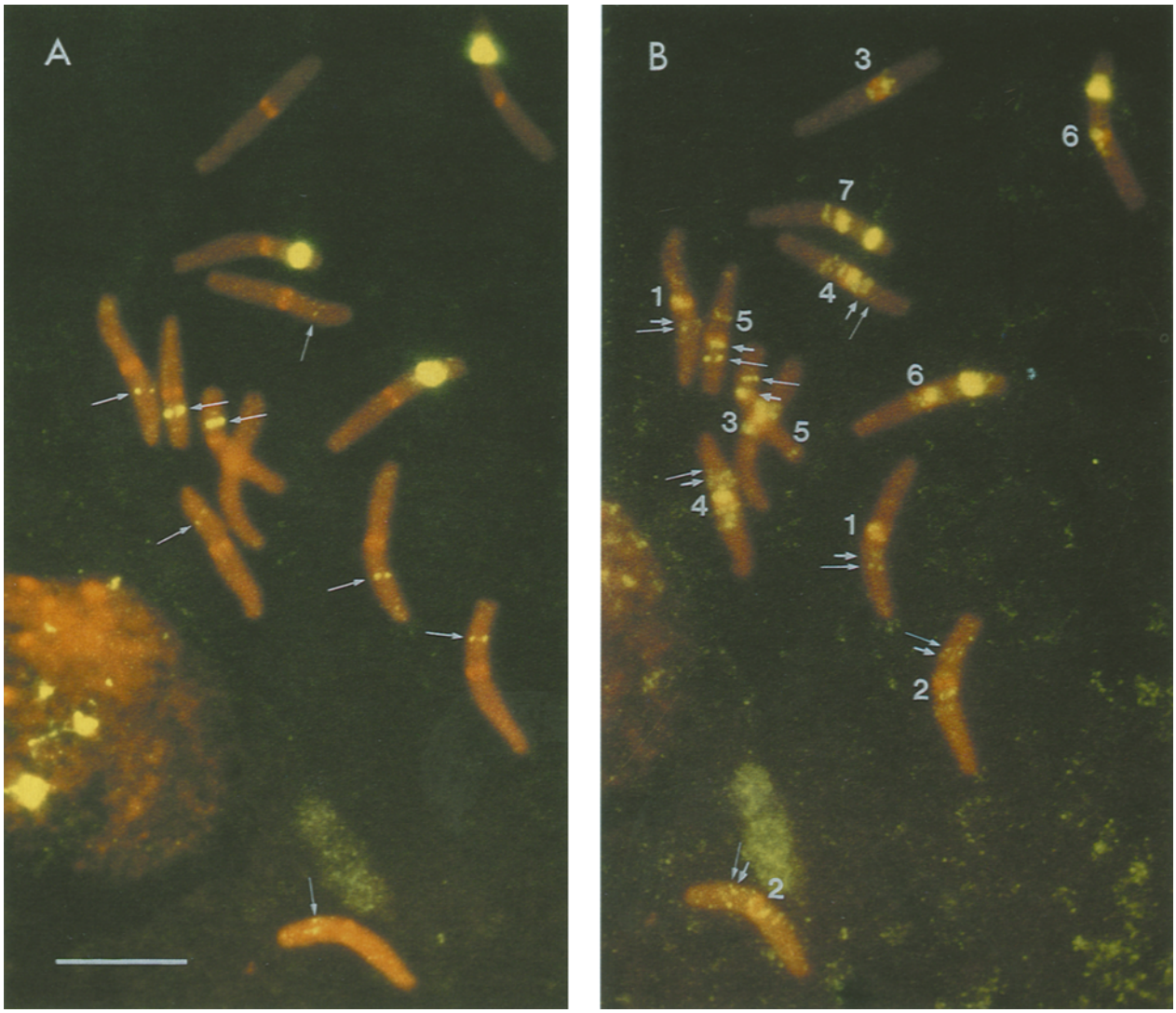

Figure 1. A Chromosomes of barley (*Hordeum vulgare* cv. Emir) at somatic metaphase after *in situ* hybridization with an rDNA probe (pTA71). Arrows indicate the 8 minor rDNA hybridization sites. One chromosome (7) is missing. **B** The same metaphase plate after reprobing with a biotin-labelled $(GAA)_7$ oligonucleotide. Overall, the hybridization pattern is similar to that produced by Giemsa C-banding, thus permitting the unequivocal identification of the chromosomes. The pTA71 hybridization sites are indicated by long arrows, and the positions of the closest GAA-band by short arrows. The numbering of chromosomes is according to the standard barley nomenclature. Bar = 10 μm.

Table 1. Fraction lengths (FLs) and relative distances from rDNA hybridization sites to closest GAA-band (in percent) for barley chromosomes 1, 2, 4, and 5

Chromosome	1 (7I)	2 (2I)	4 (4I)	5 (1I)
Fraction length (%)	38.9	48.0	51.8	39.1
SD	2.3	2.2	1.8	2.8
n	63	55	58	57
Relative distance to GAA-band (%)	10.8	9.8	18.4	27.9
SD	1.9	1.7	3.0	3.2
n	58	47	52	57

SD = standard deviation
n = number of chromosomes included in measurements

constrictions were missing. The GAA bands enabled identification of all chromosomes and individual chromosome arms in most metaphases. The hybridization signals from the first cycle of hybridization with pTA71 were still visible, but somewhat weaker.

Physical localization of the minor rDNA loci

The identification of the chromosomes based on their banding patterns enabled the localization of the three minor rDNA sites to chromosomes 1 (7I), 2 (2I), and 4 (4I), the site on chromosome 4 showing the weakest signal. All the sites were located on the arm designated the short, or the + arm (Jensen & Linde-Laursen 1992) distally to the closest GAA-band. Chromosomes with signals from 50 metaphases of cv. Emir were measured. Twenty-six metaphases had 14 identifiable chromosomes and of these, twenty-two showed all 12 hybridization sites. Based on these measurements, the FLs of the hybridization sites were 39% on chromosome 1, 48% on chromosome 2, 52% on chromosome 4, and 39% on chromosome 5 (Table 1).

The relative distances to the closest GAA-band were calculated for all chromosomes showing both rDNA- and GAA-signals after reprobing (Table 1).

Discussion

Physical localization of the microsatellite sequence

The microsatellite sequence used in this study was first described by Dennis *et al.* (1980). It occurs in long tandem repeats and is present in very high copy number in the chromosomes of wheat and barley. When used for *in situ* hybridization to barley chromosomes, this sequence produced banding patterns which however, proved unsatisfactory for reliable chromosome identification due to the low resolution of the radioactive technique used. In our study, the probe revealed clear GAA-bands corresponding to at least 27 major C-bands (Jensen & Linde-Laursen 1992) thus supporting the proposition that the GAA-bands are identical with these C-bands. The banding patterns obtained enabled an easy and unequivocal identification of all the chromosomes and the individual chromosome arms in most metaphases. Although the GAA-banding patterns on the arms of chromosomes 2 and 4 were similar, the differences in the strength of the hybridization signals permitted the correct orientation of the chromosomes.

rDNA loci in barley

rDNA loci outside the major sites on chromosomes 6 and 7 have recently been described on chromosomes 1, 2, and 5 by Leitch & Heslop-Harrison (1992) for the cultivar Sultan. We discovered an additional rDNA locus, located about 52% out on the short arm of chromosome 4. We have designated this new locus *Nor-I6* in accordance with the naming of previously published rDNA sites. We have detected these 4 minor loci in the cv. Emir, in the closely related cv. Sultan, and in the cvs. Igri and Montcalm which represent different genetic backgrounds. This suggests that these loci are present in cultivated barley in general.

The minor rDNA sites on chromosomes 1 (7I) and 5 (1I) correspond to rRNA genes on homoeologous chro-

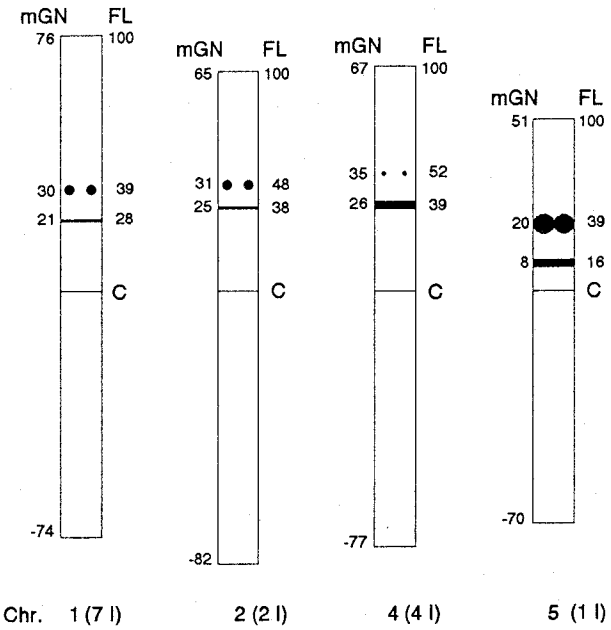

Figure 2. Idiogram of barley chromosomes 1, 2, 4, and 5 showing the physical locations of the rDNA loci (double spots) relative to the closest C-bands (transverse bars). Fraction lengths (FLs) of rDNA positions are indicated to the right of the chromosomes. The FL-values have been recalculated to milliGeNomes (mGNs) to the left of the chromosomes. Chromosome lengths are in mGNs, C-band positions are in mGNs and their FLs are based on Jensen & Linde-Laursen (1992).

mosomes in rye and wheat, as described by Leitch & Heslop-Harrison (1992), whereas the sites on the homoeologous groups 2 and 4 have not been reported in other *Triticeae* species.

The physical locations of the rDNA loci examined in this study are shown on the idiogram of Figure 2. (The well-known NORs on chromosomes 6 and 7 are omitted.) The positions of the minor rDNA sites on chromosomes 1, 2 and 5 correspond well with the positions calculated by Leitch & Heslop-Harrison (1992).

The relative distances to the closest GAA-band were calculated in order to localize the rDNA loci more precisely by using the GAA-bands as cytological landmarks. In order to establish the reliability of the GAA-band positions, we recalculated the FLs of the rDNA sites to milliGeNomes (mGN; one mGN is one thousandth of genome length, Jensen & Linde-Laursen 1992), assuming that the relative lengths of the examined chromosome arms were similar to those analysed in the study of Jensen & Linde-Laursen (1992) (Figure 2). A comparison of the relative distances between the rDNA sites and the GAA-bands found in this study (Table 1) with those of Jensen & Linde-Laursen (1992) (Figure 2) showed a very good fit for chromosomes 1 and 2 (11% and 10% respectively). However, we found longer distances on chromosomes 4 and 5 (18% versus 13%, and 28% versus 23%). The inconsistency corresponds to a physical distance of only 0.2-0.3 μm and may best be explained by errors arising when rather wide GAA- or C-bands are included in the measurements, or by difficulties in determining the positions of the centromeres, or because the assumption of similar relative chromosome arm lengths in the two barley lines is invalid.

In conclusion, the precision of the chromosomal position of a hybridization site depends on the number of measurements, the extension of the hybridization signal, and its position relative to stable cytological markers, (e.g. centromeres, telomeres, or GAA-bands). This study suggests that especially narrow GAA-bands, situated close to the hybridization site, are valuable cytological landmarks, if their positions in the karyotype in question are determined precisely.

Activity and origin of rDNA-sites

In general, only up to four normal-sized nucleoli are formed in barley, but in cv. Wong two additional micronucleoli have occasionally been noticed (Linde-Laursen 1984) indicating that at least one of the minor rDNA sites, probably that on chromosome 5, may sometimes be active. However, normally, the activity of the minor rDNA sites is probably suppressed.

It could be postulated that the minor rDNA sites do not represent rDNA genes, but arise from sequences with homology to parts of the rDNA-repeat. In *Drosophila hydei* (Huijser & Hennig 1987) and in *Anemona blanda* (Hagemann *et al.* 1993) repetitive sequence families showing homology to the rDNA repeat have been described. The repetitive sequence family from *A. blanda* diverged 16-20% from the rDNA sequence, and *in situ* hybridization with the sequence showed irregular and faint hybridization to the NORs (Hagemann *et al.* 1993). We think that all the hybridization sites reported here represent rDNA genes, or pseudogenes with high homology to rDNA genes, but sequencing data from the minor rDNA sites are required to confirm this.

It is striking that in Figure 2 the positions of the rDNA sites are rather similar on the four chromosomes, and more proximal than those on chromosomes 6 and 7. The equilocality of these minor rDNA sites may reflect their origin. One hypothesis could be that the rDNA sequences have been transferred between non-homologous chromosomes lying in Rabl-configuration. Such a mechanism has been proposed for the equilocal distribution of some highly repeated sequences (Flavell 1982). Another explanation might be that the minor rDNA sites are remnants of former NORs in an ancestral karyotype with more satellite chromosomes derived from the same primitive chromosome. The activity of rRNA genes is correlated with the length of the intergenic spacer and to the status of the methylation of cytosine residues in regulatory sequences (Flavell *et al.* 1986, Sardana *et al.* 1993). It is likely that rRNA genes in positions more proximal than those of the normally active NORs, like the minor rDNA sites, are more methylated (Moore *et al.* 1993) and more prone to be suppressed. During evolution such loci may be liable to become deleted.

The presence of many rDNA sites has also been described in other organisms. In white spruce (*Picea glauca*) ($2n = 24$), Brown *et al.* (1993) localized at least 12, and possibly 14 rDNA sites. Most of these were associated with nucleolar constrictions. Minor rDNA sites have also been described in brown trout (*Salmo trutta*) ($2n = 80$). At least 16 minor inactive rDNA sites were identified along with one pair of NORs (Pendás *et al.* 1993). However, these minor rDNA sites were associated with constitutive heterochromatin in apparent contrast to the minor sites in barley. Minor rDNA sites have also been detected in some wild *Triticeae* species. Ørgaard & Heslop-Harrison (in preparation) found 18 rDNA sites in *Psathyrostachys stoloniformis* C. Baden (2×), and we have observed minor rDNA sites in *Hordeum marinum* Huds. ssp. *gussoneanum* (Parl.) Thell. (4×) and *H. brevisubulatum* (Trin.) Link ssp. *turkestanicum* (Nevski) Tzvel. (4×) (unpublished observations). Inactive, minor rDNA loci may be more common and widespread than previously appreciated (Linde-Laursen *et al.* 1992).

Conclusions

We have physically mapped the two major and the four minor rDNA loci in barley through *in situ* hybridization

by reprobing with a microsatellite sequence hybridizing to the positions of the C-bands, thus enabling chromosome identification. We are presently isolating clones containing the GAA sequence in order to make longer hybridization probes and perform two-colour fluorescence *in situ* hybridization (FISH) with the microsatellite sequence together with other probes. This strategy is easier, more direct, and faster than the combination of Giemsa C-banding following *in situ* hybridization (Leitch & Heslop-Harrison 1992) or the approach used in this study. For probes which hybridize close to a distinct C-band this will also permit more accurate physical mapping. Fluorescent *in situ* hybridization with the GAA sequence may be an attractive alternative to the C-banding technique for chromosome identification in barley.

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