2 Materials and Methods

2.1 Sequence organization of barley centromeres

2.1.1 BAC library screening

A BAC library of genomic DNA from *H. vulgare* L. cultivar Morex (established at Clemson University) containing 313,344 clones (about 6.3 times covering the barley genome; Yu *et al.* 2000), was transferred onto Hybond N^+ filters (Amersham). Treatment of the filters, hybridization and washing conditions were as described (Nizetic *et al.* 1991; Hoheisel *et al.* 1993). Of ten BAC clones which hybridized with the integrase region (pGP7) of the polyprotein gene of *Ty3*/*gypsy*-like retrotransposon *cereba* (Presting *et al.* 1998) labelled with ^{32}P-dCTP using a random primer extension kit (Amersham) according to Feinberg and Vogelstein (1983), only one (03J24, now called BAC 7) showed after fluorescent in situ hybridization (FISH) positive signals exclusively at the centromeric regions of all barley chromosomes. **(done by G. Presting and W. Michalek)**

2.1.2 Chromosome preparation, Probe labelling, Fluorescent *in situ* hybridization (FISH) and Genomic *in situ* hybridization (GISH)

Metaphase spreads from root tip meristems of the barley line MK 14/2034 (characterized by two homozygous reciprocal translocations between chromosomes 3H/4H and 7H/5H) were prepared as described (Presting *et al.* 1998). Briefly, root tip meristems were placed in distilled water at 0 °C for 16-24 h, fixed in 3:1 ethanol:glacial acetic acid for 24 h and washed in water. They were digested for 30-60 min in an enzyme mix consisting of 2.5% pectolyase and 2.5% cellulase Onozuka R-10 in 75mM KCl, 7.5 mM EDTA at pH 4.5, squashed in 45% acetic acid and air-dried.

For FISH, BAC 7 DNA was isolated using a QIAGEN Plasmid Mini Kit (100) and labelled with rhodamin-5-dUTP using a nick translation kit (Roche Biochemicals) according to manufacturer's instructions.

The primers $(AGGGAG)_4$ and $(CTCCCT)_4$ representing the most frequent motif within the G+C-rich domain outside the *cereba* elements of the BAC 7 insert, were amplified without additional template sequence and biotin-labelled by PCR according to Ijdo *et al.* (1991). Briefly, the PCR-mix was composed of 10x PCR buffer (without $MgCl_2$), 0.3 mM $MgCl_2$, 0.2 mM dATP, dGTP, dCTP, 0.1 mM dTTP, 25 nmol rhodamin-5-dUTP (Boehringer Mannheim), 0.1 μM of each primer and 2 units of bioTaq polymerase (biomaster). Amplification consisted of ten cycles (each cycle: 1 min at 94 °C, 30 sec at 55 °C, 1 min at 72 °C), followed by thirty cycles (each cycle: 1 min at 94 °C, 30 sec at 60 °C, 90 sec at 72 °C) and last step of 5 min at 72 °C.

For GISH, genomic barley DNA, isolated according to Bernatzky and Tanksley (1986) was labelled with biotin using a nick translation kit (Roche Biochemicals) according to manufacturer's instructions and precipitated. For precipitation: one-tenth volume of 3M NaAc (pH 5.2) and 2.5 volumes of (-20 °C) 96% ethanol were added to the nucleic acid solution and placed on ice for 30 min. Then, the sample was centrifuged at 13,000 rpm for 30 min, the supernatant was removed, the precipitate air-dried and dissolved in distilled water. The hybridization mixture consisted of 0.1 μg labelled genomic DNA/slide and 1 μg of unlabelled BAC 7 DNA/slide, 50% formamide, 2x SSC and 10% dextran sulphate.

Slides for FISH were washed for 2 x 5 min in 2x SSC, dehydrated in ethanol series (70, 90, 96%, 3 min each) and air-dried. Fifteen microliters of hybridization mixture (80 ng labelled DNA/slide, 50% formamide, 2x SSC, 10% dextran sulphate) were applied per slide, covered by 24 x 32 mm coverslip, denatured at 80 °C for 2.5 min and incubated for hybridization at 37 °C overnight in a moisture chamber. Post-

hybridization wash was done in 2x SSC for 2 x 5 min at room temperature. Slides with directly (rhodamin) labelled probes were mounted in Vectashield (Vector Laboratories) supplemented with 2.0 µg/ml DAPI as a counterstain. Biotin-dUTP labelled probes were detected by Texas Red-conjugated avidin (Vector Laboratories, Burlingame, CA, USA) and signals were amplified by biotinylated goat-antiavidin (Vector Laboratories) and Texas Red-conjugated avidin (Fransz *et al*. 1996). After signal amplification, slides were mounted in Vectashield, as described above. GISH was performed according to protocol described above for FISH.

2.1.3 BAC size determination

The size of the BAC 7 clone was measured by pulsed field gel electrophoresis (PFGE) using the CHEF-DR® II electrophoresis system (Bio-Rad) with a 5 sec pulsed time (5V/cm) for 15 h on a 1% agarose gel (GIBCOBRL) at 14 °C in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). A λ/*Hind* III ladder (MBI Fermentas) was used as molecular weight marker.

2.1.4 Restriction digests, Agarose gel electrophoresis and Southern blot analysis

For restriction analysis, aliquots containing 70 ng of BAC 7 DNA were completely digested for 3 h at 37 °C with ten different restriction endonucleases (*Bgl* II, *Bst* XI, *EcoR* I, *Hind* III, *Kpn* I, *Not* I, *Pst* I, *Sal* I, *Sfu* I, *Xba* I) and 20 double combinations. The digestion products and the molecular weight markers Smartladder (EUROGENTEC) and Gene Ruler™ DNA Ladder Mix (MBI Fermentas) were electrophoresed on 0.8% agarose gels (GIBCOBRL, Life Technologies) in 1x TBE buffer at 78 V for 4 h.

To perform Southern blot analysis, single or double digests of BAC 7 DNA with the restriction enzymes *EcoR* I, *Hind* III, *Pst* I, *Not* I, *Sal* I were carried out. The fragments were separated on 1% agarose gels and blotted onto a Hybond-N^+ nylon membrane (Amersham LIFE SCIENCE) in 20x SSC solution. The DNA was fixed on the membrane by exposure to UV light for 3 min. Prehybridization and hybridization were performed overnight at 68 °C and 58 °C, respectively, in 5x SSC, 0.1% (w/v) N-laurosylsarcosine, Na-salt (Sigma), 0.02% (w/v) SDS and 0.5% (w/v) blocking reagent (Boehringer Mannheim). As probes were used pBeloBAC 11 (vector) and the following inserts of subclones of the λ9 clone (accession number AF078801, see Presting *et al.* 1998), which represent parts of the retrotransposon *cereba*: pGP7 (1.5 kb, RNase H + integrase domain), pGP12 (1.6 kb, gag + RNA binding domain), pGP33 (1.6 kb, including 182 bp homologous to the barley variant of CCS1; see Aragón-Alcaide *et al.* 1996), pGP5 (1.1 kb, reverse transcriptase domain) and pGP13 (0.46 kb, protease domain). The pGP inserts were obtained by digestion of the subclones pGP7 and pGP5 with *Xba* I and *Hind* III and of pGP12, pGP13 and pGP33 with *EcoR* I and *Hind* III and extraction from gels using a QIAEX Kit (QIAGEN). Probes were labelled using a Dig-high prime Kit (Boehringer Mannheim), according to the supplier´s instructions. After hybridization, the membrane was washed twice in 2x SSC, 0.1% SDS for 5 min at room temperature and twice in 0.1x SSC, 0.1% SDS for 5 min at 58 °C. The DNA-DNA hybrids were detected by chemiluminescence with the CSPD® Kit (Boehringer Mannheim). Prior to reuse, the membrane was stripped by boiling in 0.5% SDS. **(These experiments were started by K. dos Santos and R. ten Hoopen, and continued and finished by S. Hudakova.)**

2.1.5 Subcloning, Shotgun sequencing and Data analysis

BAC 7 DNA was sonicated and fragments (~550 bp) were subcloned into the pBluescript II SK- vector (Stratagene) and sequenced using an ALFexpress (Pharmacia Biotech) or an ABI Prism 377 (Perkin Elmer) DNA sequencer at the IPK **(done by G. Presting and W. Michalek)**. A sequence of ~3.9 kb, constituting the central part of a *Hind* III fragment of ~4.8 kb, could not be sequenced completely even by specialized Biotech companies (SEQLAB GmbH Göttingen). The shotgun-sequencing data were analysed with the Sequencher 3.1.1. software (Gene Codes). The resulting contigs were compared with the GenBank entries for the λ9 clone and the cereal centromeric sequence (CCS1) (position 1-260) of the Hi-10 clone derived from *B. sylvaticum* (U52217) at NCBI using the BLASTN homology search software (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). The program TAIR BLAST™ Similarity Search (http://arabidopsis.org/blast/) was used for comparison of the BAC 7 insert sequence with other plant sequences of the GenBank database. The nucleotide sequence of BAC 7 clone has been deposited to GenBank under the accession numbers AY 040832 and AY 040833.

2.2 Reconstruction and investigation of barley karyotypes with recombinantly elongated chromosome arms

2.2.1 Plant material, Chromosome preparation, Giemsa N-banding and Fluorescent *in situ* hybridization

Two homozygous translocation lines of *Hordeum vulgare* var. Bonus, T1-6y(1S-6L) (=P1), see Ramage (1975) and T1-7f(1S-7L) (=P2), see Ramage (1971), were crossed with each other as done by Tuleen and Gardenhire (1974). The F_2 individuals were screened for cytotypes with an elongated chromosome arm (**Figure 5**, see p. 32).

Since chromosome 1 was involved in both translocations, in the F_1 meiotic recombinations were expected to occur between the homologous regions of chromosome 6^1 of karyotype P1 and chromosome 1^7 of karyotype P2. This would generate a karyotype with chromosome $6^{1/7}$ (designated as A) and a normal karyotype (designated as W) (**Fig. 5b, c**). Four more cross combinations between different translocation lines were made and the F_2 individuals were examined for recombinantly elongated chromosome arms (**Figure 6**, see p. 33). **(original crosses were done by G. Künzel)**

Root tips of F_2 seedlings were pre-treated with ice-water for 16 h to accumulate metaphase cells, fixed in 3:1 ethanol:glacial acid (v/v) for three days at room temperature, stained in 1% acetocarmine for 1 h and squashed in a drop of 45% acetic acid. Individuals with an elongated chromosome were identified by Giemsa N-banding according to Georgiev *et al*. (1985). Briefly, slides were incubated in 45% acetic acid for 10 min in a water bath at 60 °C and air-dried. Afterwards, they were incubated in phosphate buffer (1M NaH_2PO_4) at 92 °C for 2 min, stained with 3% Giemsa (Merck) solution {110 ml of Sörenson's buffer [508 ml of 0.9% KH_2PO_4 (w/v) and 492 ml of 1.2% Na_2HPO_4 (w/v)] + 3.3 ml of Giemsa} for 1 h at room temperature, washed in distilled water and mounted in euparal. The same procedure was used to define the karyotypes in F_3 as well as of F_2 individuals from four further crosses between different translocation lines (**Figure 6**, see p. 33).

For the preparation of meiocytes, spikes of the plants containing elongated chromosome arms were fixed as for root tips, gently squashed in a drop of acetocarmine and stained with DAPI (1µg/ml).

For FISH, the subtelomeric 119 bp tandem repeat HvT01 (Belostotsky and Ananiev 1990) was used as a probe. The probe was labelled by rhodamin-5-dUTP via PCR amplification from 100 ng of genomic barley DNA. The PCR-mix was composed

of 10x PCR buffer (without $MgCl_2$), 0.3 mM $MgCl_2$, 0.2 mM dATP, dGTP, dCTP, 0.1 mM dTTP, 25 nmol rhodamin-5-dUTP (Boehringer Mannheim), 0.3 µM of each primer (5'CGAAACTCGCATTTTTGGCC3' and 5'AGAGTTCCCGTAACCGGCCC3', positions 2-21 and 118-99 of the basic sequence unit of HvT01) and 2 units of bioTaq polymerase (biomaster). Thirty-five cycles were run (1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C). Fifteen microliters of hybridization mixture (80 ng labelled DNA/slide, 50% formamide, 2x SSC, 10% dextran sulphate) were applied per slide, covered by 24 x 32 mm coverslip, denatured at 80 °C for 2.5 min and incubated for hybridization at 37 °C overnight in a moisture chamber. Post-hybridization wash was done in 2x SSC for 5 min at room temperature and quick wash in distilled water. Drained slides were mounted with 10 µl Vectashield (Vector) containing 1 µg/ml DAPI.

2.2.2 Feulgen staining, Chromosome arm and spindle lengths measuring

Incomplete sister-chromatid separation, the occurrence of micronuclei and the length of chromosome arm and spindle axis were studied on Feulgen-stained lateral roots of seedlings of the normal karyotype, of the line MK 14/2034 (homozygous for the two reciprocal translocations T3-4ae and T1-7an, see http://wheat.pw.usda.gov/ggpages/Barley_physical/Idiograms/) and of plants heterozygous (AP1, AP2) and homozygous (AA) for recombinantly elongated chromosomes. The root tips were fixed as described above, hydrolyzed in 1N HCl (63 °C, 11 min) and stained in Schiff´s reagens (1 h). The meristem tissue was gently squashed in 1% acetocarmine to prevent disruption of cells and mounted with euparal. The length of chromosome arm and spindle axis (distance from pole to pole) at anaphase and telophase were measured with the software MicroMeasure 3.3 image analysis (http://www.colostate.edu/Depts/Biology/MicroMeasure/).