Chromosome analysis in barley:

DNA composition and organization of centromeres

and

the upper chromosome size limit

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Content

List of abbreviations

A	recombinant karyotype with elongated chromosome arm
BAC	bacterial artificial chromosome
BCS2	barley variant of the cereal centromere sequence1
CCS1	cereal centromere sequence1
cereba	*ce*ntromeric *re*troelement of *ba*rley
DAPI	4’,6-Diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
FISH	fluorescent in situ hybridization
GISH	genomic in situ hybridization
L	long chromosome arm
LTR	long terminal repeat
NOR	nucleolar organizer
PBS	primer binding site
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PPT	polypurine tract
P1, P2	parental lines
RB	RNA binding site
rDNA	ribosomal DNA
rpm	revolutions per minute
S	short chromosome arm
SD	standard deviation
SDS	sodium dodecyl sulphate
Tris	Tris-(hydroxymethyl)-aminomethan
UV	ultraviolet
W	normal karyotype, wild-typ

1 Introduction

This dissertation consists of two parts, 'DNA composition and organization of centromeres' and 'The upper chromosome size limit', both having barley as the common subject.

Barley (*Hordeum vulgare* L.) is an annual cereal of the family Gramineae (grass family), classified in the division Magnoliophyta , class Liliopsida, order Cyperales, family Gramineae. Indications from archaeological remains in the Near East, corresponding geographically to a region extending from Israel through Syria, southern Turkey into Iraq and Iran, suggest that the crop was domesticated about 10,000 years ago from its wild relative *Hordeum spontaneum* (Salamini *et al.* 2002).

Barley is nowadays used commercially for animal feeding, to produce malt for beer and whisky production and for human food applications. It is the fourth most important cereal crop in the world after wheat, rice and maize.

The annual world production of barley (1996-2001) is about 142 million tonnes (http://apps.fao.org/page/form?collection=Production.Crops.Primary&Domain=Production&servlet=1&language=EN&hostname=apps.fao.org&version=default). Barley has a wide range of cultivation and matures even at high altitudes, since its growing period is short, however, it cannot withstand hot and humid climates.

1.1 The centromere: function and structural organization

The centromere is a highly specialized structure of all eukaryotic chromosomes required for correct transmission of the nuclear genetic information from cell to cell and from generation to generation. On monocentric chromosomes it is microscopically recognizable as the primary constriction. It has a central stage role during nuclear

division and fulfils several essential functions. Centromeres are responsible for sister chromatid cohesion until anaphase, represent the site for kinetochore assembly and for attachment of mitotic and meiotic spindle fibres. They are necessary for segregation of sister chromatids into daughter nuclei during mitosis and meiosis II and of homologous chromosomes during meiosis I, and are involved in cell cycle checkpoint control via 'anaphase promoting complex' (for review see Choo 1997; Maney *et al.* 1999).

1.1.1 Centromeric DNA

Although the centromere function is highly conserved among eukaryotes, centromeric DNA sequences are considerably variable between species. A functional centromere of *Saccharomyces cerevisiae* (budding yeast) needs only a 125-bp sequence organized into three elements: CDE I (8 nucleotides), CDE II (an AT-rich ~80-nucleotide sequence) and CDE III (a conserved sequence of 26 nucleotides) (Clarke and Carbon 1985; Hieter *et al.* 1985; Clarke 1990). In *Schizosaccharomyces pombe* (fission yeasts), the central core (*cen1, cen2, cen3*) and at least one block of repeated elements (K-type repeats), has been shown to be essential for correct centromere function (Takahashi *et al.* 1992; Baum *et al*. 1994).

The centromere of higher eukaryotes is usually embedded within large blocks of heterochromatin (White 1973; Choo 1997) characterized by the presence of tandemly repeated DNA in long arrays.

1.1.1.1 Tandem repeats

Many satellite or other tandem repeats with characteristic chromosomal location have been identified and cloned from different organisms. Centromere-associated repeats may represent a considerable fraction of the genomic DNA. Repetitive AT-rich

DNA seems to be a common feature of centromeric DNAs in several organisms such as *S. cerevisiae* (AT-rich CDE II element, Clarke and Carbon 1985; Clarke *et al.* 1993), *Drosophila* (AATAT satellite, Murphy and Karpen 1995; Sun *et al.* 1997), human and other mammals (alphoid DNA with an AT-rich ~171 bp tandem repeat, Manuelidis 1978a, 1978b; Mitchell *et al.* 1985; Willard 1985; Choo *et al.* 1991). Although alphoid satellites are conserved among primates, a considerable variability in sequence became evident even between centromeres of individual human chromosome pairs (Willard 1985; Choo *et al.* 1991; Choo 1997). Similar chromosome-specific variants have been identified in the centromeric minor satellite of the mouse (Kipling *et al.* 1991; 1994).

Various centromere-specific repeats were isolated also from different plant species. For instance *Arabidopsis* centromeres contain tandem arrays of the 180 bp repeat (Martinez-Zapater *et al.* 1986; Simoens *et al.* 1988; Maluszynska and Heslop-Harrison 1991). Species-specific satellite sequences organized in tandem repeats were found also in cereals, e.g. RCS2 in rice (Dong *et al.* 1998), CentC in maize (Ananiev *et al.* 1998), TrsD in rice (Kumekawa *et al.* 2001), the TaiI family in wheat (Kishii *et al.* 2001), CentO in rice (Cheng *et al.* 2002), the pBoKB1 and pBcKB4 repeats in *Brassica* (Harrison and Heslop-Harrison 1995) and the satellite repeat pBV1 in *Beta vulgaris* (Schmidt and Metzlaff 1991). Nevertheless, for some plants (such as field bean and *Tradescantia*) no centromere-specific tandem repeats could be detected (Houben *et al.* 1996).

1.1.1.2 Other centromeric repeats

In addition to the tandemly repeated DNA, a number of other repeat sequences have been found at or near centromeres, which are either genome-wide dispersed or

mainly restricted to centromeric regions, often representing complete or truncated mobile genetic elements, which can be divided into two major groups:

class I including retroviruses (found only in animals); long terminal repeat (LTR) - containing retroelements of the *Ty1*/*copia* and *Ty3*/*gypsy* group, differing in the order of genes encoding their proteins) and non-LTR retrotransposons (e.g. LINE and SINE elements), which transpose by reverse transcription of RNA intermediate, and **class II** (e.g. *Ac*, *En*/*Spm*), which transpose by an excision/insertion mechanism (Kumar and Bennetzen 1999).

In many cases retrotransposons are widely dispersed e.g. *Ty1*-*Ty4* elements inserted into euchromatic regions of *S. cerevisiae* (Boeke 1989; Voytas 1996), *copia* elements present in both eu- and heterochromatic regions in *Drosophila* (Levis *et al.* 1980; Mount and Rubin 1985; Carmena and Gonzales 1995), *Ty1*/*copia* elements in plants (Flavell *et al.* 1992; Brandes *et al.* 1997; Heslop-Harrison *et al.* 1997). Ta elements of *Arabidopsis* (Konieczny *et al.* 1991), the Tnt1 element of tobacco (Grandbastien *et al.* 1997), BARE-1 of barley (Manninen and Schulman 1992; Suonemi *et al.* 1996; 1997) and *Ty/copia* elements in *Vicia* (Pearce *et al.* 1996) and onion (Pich and Schubert 1998) are mainly located in euchromatic regions. Also other elements such as LINEs and SINEs show dispersed chromosomal distribution in human and other mammals (Smit 1996; 2000) and also in plants (Kumar and Bennetzen 1999). Mostly these elements are present in low amount or absent from specific chromosome regions, e.g. centromeres, interstitial and terminal heterochromatin, and rDNA sites (Kumar and Bennetzen 1999). However, there are some exceptions, for example, non-LTR retrotransposon elements *I*, *F*, *G*, and *Doc* are present in the centromeric regions of *Drosophila* chromosomes (O'Hare *et al.* 1991; Pimpinelli *et al.* 1995). The LTR retrotransposon Athila is clustered mainly within pericentromeric heterochromatin (Pélissier *et al.* 1995; 1996) and occupies (together with 180 bp satellite) the centromeric regions of all five

Arabidopsis chromosomes (Pélissier *et al.* 1996; Fransz *et al.* 1998; 2000; Heslop-Harrison *et al.* 1999). Members of the *Ty3*/*gypsy* group of retrotransposons are accumulated within the centromeres of cereals (see below).

1.1.1.3 Centromeric sequences of cereals

Two centromeric sequences were described for cereals. One is the ‚cereal centromeric sequence' (CCS1) family of *Brachypodium* that also occurs in wheat, rye, barley, maize and rice centromeres (Aragón-Alcaide *et al.* 1996) and the other is the *Sau3A9* sequence of sorghum which also hybridized to the primary constrictions of the above species (Jiang *et al.* 1996). Using a barley homologue of *Sau3A9* as a probe, a λ clone (#9) from a genomic library was detected containing a ‚cereba' element (centromeric retroelement of barley). The λ9 clone possesses a complete polygene, with high similarity to the *Ty3*/*gypsy* group of retrotransposons, of which *Sau3A9* represents the integrase encoding region, and flanking sequences similar to CCS1, supposed to represent LTRs of *cereba.* This element hybridized to all barley centromeres (Presting *et al.* 1998). Meanwhile, further conserved sequences representing parts of *gypsy*-like retroelements were found within the centromeres of several cereals such as CentA in maize (Ananiev *et al.* 1998); pHind22 in sorghum, wheat, maize and rye (Miller *et al.* 1998a); RCS1 in rice, rye, barley, sorghum and maize (Dong *et al.* 1998); RCB11 in rice and crwydryn in oats and rye (Nonomura and Kurata 1999; Langdon *et al.* 2000); RIRE7 in rice (Kumekawa *et al.* 1999; Kumekawa *et al.* 2001; Nonomura and Kurata 2001); R11H in wheat (Fukui *et al.* 2001); CRR in rice (Cheng *et al.* 2002). *Gypsy*-like elements (pBv26 and pBp10) were found even within centromeres of dicotyledonous Beta species (Gindullis *et al.* 2001).

The very low conservation of centromeric DNA sequences indicates, that their functional importance is at least controversial, the more so since for several species neocentromeric activities at non-centromeric chromosomal positions have been reported (Depinet *et al.* 1997; du Sart *et al.* 1997). Therefore, it is suggested, that the centromere location might be regulated epigenetically (Vig 1994; Karpen and Allshire 1997).

1.1.2 The kinetochore

The kinetochore is a protein complex associated with eukaryotic centromeres. It plays an important role in interactions of centromeres with the spindle microtubules, in chromosome movements during nuclear divisions, and in the checkpoint (metaphase-anaphase transition) control (Rieder and Salmon 1998; Maney *et al.* 1999). More than 20 proteins associated with the centromere/kinetochore structure have been identified in non-plant organisms. They can be classified into two groups: i) structural proteins (e.g. CENP-A, CENP-B, CENP-C and CENP-H, Sugata *et al.* 2000; Fukagawa *et al.* 2001; for review see Choo 1997), which are constitutively present at centromeres, and ii) passenger proteins (e.g. the BUB family, the MAD family, ZW10, CENP-E, CENP-F and others; Earnshaw and Bernart 1990; Rattner *et al.* 1993; Liao *et al.* 1995; Taylor and McKeon 1997; Starr *et al.* 1997; Yen *et al.* 1991; Yao *et al.* 2000; Saffery *et al.* 2000), which transiently occur at centromeres during nuclear division. Several centromere proteins have been found to be evolutionarily conserved within eukaryotes (Dobie *et al.* 1999). For instance, at least partial homology was found between the yeast Mif2 and the mammalian CENP-C (Earnshaw and Rothfield 1985; Brown 1995) and a putative homologue of maize (Dawe *et al.* 1999). *Drosophila melanogaster* ZW10 homologues are present in *C. elegans*, *A. thaliana*, mice and human (Starr *et al.* 1997). Putative homologs of yeast SKP1 kinetochore protein were found in *Vicia faba* and

barley, and of yeast CBF5p in barley (ten Hoopen *et al.* 2000). Furthermore, cross reactivity was observed for human anti-CENP-E (Yen *et al.* 1991) and anti-CENP-F antibodies (Rattner *et al.* 1993) with kinetochores of *Vicia faba* and barley (ten Hoopen *et al.* 2000).

The high conservation of kinetochore protein sequences, in contrast to the low conservation of centromeric DNA, between remotely related eukaryotic groups suggests that also their functions might be evolutionary conserved.

1.1.3 Aims of the work on barley centromeric DNA

At the beginning of this work large scale sequences and organization of centromeric DNA was not known for plant subjects. Presting *et al.* (1998) have shown, that the sequence of barley λ9 clone possesses in addition to an apparently complete *cereba* element also BARE retroelement sequences, which are dispersed along the chromosome arms of barley (Manninen and Schulman 1992). Furthermore, the *Dra* I restriction pattern of λ9 differed from that of genomic DNA when probed with the barley homologue of *Sau3A9*. For these reasons, it was assumed that this clone might contain either sequences of a centromere-border or represents a chimeric insert not really representative for barley centromeres. Therefore, a genomic BAC library has been screened with the barley homologue of *Sau3A9.* A BAC clone (03J24, here BAC 7) was found to yield FISH signals exclusively at all barley centromeres, and a hybridization pattern comparable to that of genomic DNA after digestion with *Dra* I and Southern hybridization with pGP7 (a barley homologue of the centromere-specific *Sau3A9* element of sorghum) and BCS2 (barley variant of the *cereal centromere sequence*1). This BAC clone was used for further investigations. After shotgun sequencing the aims of this part of the work were:

1. to sequence BAC 7 fully and to align the sequence components for establishing sequence organization characteristic for barley centromeres;
2. to prove whether the CCS1-like sequence belongs to the retroelement *cereba*, and to find out whether other centromere-specific sequences are associated with *cereba*;
3. to compare these sequences with that of other cereal centromeres.

1.2 Chromosome size limitations

The size of chromosomes may vary considerably (from <1 to >20 μm) within and between natural karyotypes. However for theoretical reasons both, lower and upper size limitations must be considered. The question is how such limits are defined.

1.2.1 Lower limit of chromosome size

Indications for a lower size limit for stable chromosome transmission especially during meiosis come from observations made on minichromosomes of yeast, mammals, insects and plants. It was suggested, that in most cases chromosomes should contain ≥ 1% of the host´s genome size for mitotic and clearly more for perfect meiotic stability (for review see Schubert 2001). Possibly, a certain amount of chromatin flanking a centromere is required e.g. for H3 phosphorylation (Houben *et al.* 1999; Manzanero *et al.* 2000) as a lateral support for correct segregation (Schubert 2001). It was recently shown, that in fission yeast flanking heterochromatin is required for cohesion between sister centromeres (Bernard *et al.* 2001). These observations have critical implications for the construction of stable artificial chromosomes.

1.2.2 Upper limit of chromosome size

During nuclear divisions, chromosomes have to be distributed equally to the daughter cells. During anaphase of mitosis and meiosis II chromatids and during meiosis I chromosomes are pulled by spindle fibres attached to centromeres toward the opposite poles of the spindle axis. Later in anaphase a further spindle elongation takes place increasing the distance between poles (Armstrong and Snyder 1989; Hoyt and Geiser 1996). The extension of the spindle is presumably genetically determined (Ming and Hong 2001) although it may vary between specific tissues. Therefore, the extent of spindle axis might be a parameter to determine the upper size limitation for chromosomes.

In *Nicotiana*, abnormally (up to 15-fold) elongated 'megachromosomes' which occured in a few cells of interspecific hybrids (Gerstel and Burns 1966; 1976) could not pass as intact chromosomes from cell to cell, but were broken by the cell plate and yielded chromosome breakage, fragments, dicentrics, rings, anaphase bridges and chromatin elimination. Only the ability to form such megachromosomes was transmitted.

Later on, it was found for *Vicia faba*, that the length of longest chromosome arm must not exceed half of the average length of the spindle axis at telophase (Schubert and Oud 1997). Chromosomes with arms recombinantly elongated beyond this border led to incomplete separation of sister chromatids. As a consequence, breakage of non-separated sister chromatid arms, mediated by the newly forming cell wall during mitosis, caused micronuclei representing chromatin deletions. Viability and fertility of individuals decreased proportionally with the increase of chromosome arm length above half of the average spindle axis dimension, presumably due to a significant increase in apoptotic cells compared to wild-type meristems which is caused by chromatin

deletions and decreases the amount of cells available for tissue differentiation (Schubert *et al*. 1998a).

In *Drosophila*, an abnormally long chromosome C(2)EN with a nearly doubled length of both arms due to both homologs of chromosome 2 sharing a single centromere, caused a ten-fold increase in errors (3.3%) during syncytial embryonic divisions as compared to control embryos (0.3%) (Sullivan *et al*. 1993). This became manifested by chromatin lagging on the metaphase plate, delay of anaphase and final removal of the corresponding nuclei from the population of syncytial nuclei into the inner embryo. Interestingly, in the larval neuroblast cells, the sister chromatids of compound chromosome arms were cleanly separating from each other during late anaphase, most probably because the spindle is longer in the neuroblast cells than in embryonic syncytial nuclei. Although the observed frequency of syncytial mis-division had no obvious impact on viability and fertility of the carrier organism, it seems possible that longer arms might have deleterious effect by further increasing the number of mis-divisions. This indicates that too long chromosome arms may interfere with nuclear divisions also in non-plant organisms.

1.2.3 Aims of the work on upper limit for chromosome arm length in barley

On the basis of previous data, that half of the average length of the spindle axis at telophase defines the upper tolerance limit for chromosome arm length in the field bean, *Vicia faba* (Schubert and Oud 1997), the aims of the second part of this work were:

1. to analyse barley cytotypes with recombinantly elongated chromosome arms as to:
    - mitotic and meiotic spindle axis length
    - separation of sister chromatids into daughter nuclei and formation of micronuclei during mitosis and meiosis

- the impact of elongated chromosome arm(-s) on phenotype and fertility of the plants

2. to test whether the upper tolerance limit for chromosome arm length defined by half of the spindle axis length holds true as a general rule also for other organisms, in this case the monocot barley

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/).

3 Results & Discussion

3.1 DNA sequence composition and sequence organization of barley centromeres

3.1.1 Isolation and characterization of the centromere-specific BAC clone

A genomic barley BAC library was screened with pGP7, a plasmid subclone of the λ9 clone, which is highly homologous to the integrase region of the polyprotein gene of *Ty3*/*gypsy* group retrotransposons. Ten clones were selected, but only one of these (BAC 03J24 later on called BAC 7) showed a positive FISH signal exclusively at the centromeric regions of all barley chromosomes (**Figure 1a**). The other nine BACs showed additional dispersed signals along the chromosome arms and a *Dra* I restriction pattern different from that of genomic DNA when probed with pGP7 (the barley homologue of *Sau3A9*) and BCS2 (the barley homologue of the ‚cereal centromeric sequence' family; Aragón-Alcaide *et al*. 1996). It was assumed that these clones might contain disperse repetitive sequences of a centromere-border, or they are chimeric and therefore not representative for barley centromeres. BAC 7 yielded a hybridization pattern similar to that of genomic DNA after digestion with *Dra* I and Southern hybridization with pGP7 and BCS2 **(done by G. Presting and W. Michalek)**. To determine the size of BAC 7, its DNA was isolated, digested with *Not* I and *Xho* I, respectively, and separated by PFGE (**Figure 1b**). Digestion with *Xho* I yielded only one band corresponding to the linearized plasmid (~30 kb), while *Not* I yielded two fragments, one (~6.9 kb) comprising most of the vector and the other one (~23 kb) the insert flanked by short stretches of vector DNA at both sides. Therefore, the insert size was estimated to be ~22,500 bp, which together with the vector pBeloBAC 11 (7,507 bp) constitute BAC 7.

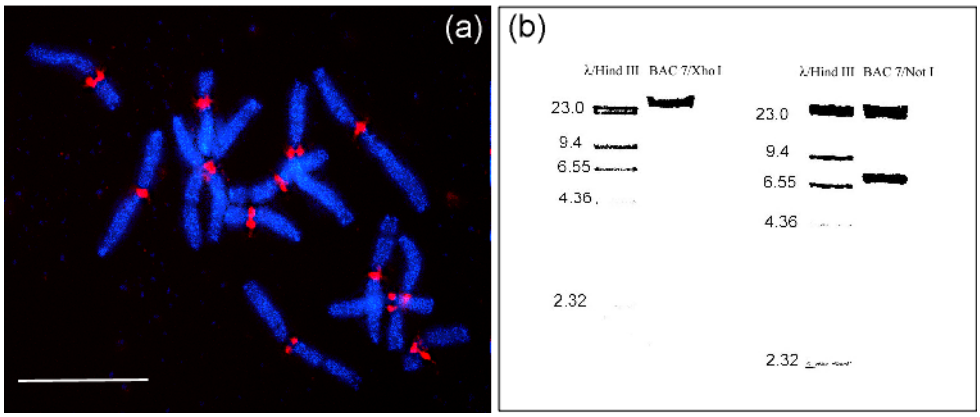

Figure 1: FISH with BAC 7 as probe on barley chromosomes **(a)**, and size determination of BAC 7 by PFGE **(b)**.

(a) BAC 7 labelled with rhodamin-5-dUTP yielded signals exclusively at the centromeres of all barley chromosomes of karyotype MK 14/2034; Bar = 10 µm

(b) BAC 7 DNA linearized by *Xho* I (left) and digested by *Not* I (right); *Not* I yielded a vector fragment (6.9 kb) and the insert (23 kb) flanked by minor parts of the vector.

3.1.2 Sequencing and restriction fragment mapping of BAC 7

Shotgun sequencing of 150 subclones of BAC 7 with an average size of 550 bp was performed **(done by G. Presting and W. Michalek)**. Because the occurrence of repetitive sequences was to be expected for the insert of BAC 7 and sequencing and alignment into contigs is difficult for such sequences, a restriction map of BAC 7 was constructed in parallel. For that purpose, DNA of BAC 7 was completely digested with 10 restriction enzymes and 20 pairwise combinations and electrophoresed on agarose gels. Southern blots were hybridized consecutively with five subclones of λ9 as well as with the vector pBeloBAC 11 as probes **(started by K. dos Santos and R. ten Hoopen)**. A compilation of the resulting fragments is given in **Table 1**. As expected, all fragments per digest amounted to approximately 30 kb, the size of the entire BAC 7. Double or triple bands were determined by comparing band intensities to that of the molecular weight markers. The restriction map has been designed manually by

assembling restriction fragments from single and multiple digests in comparison with the sequence alignment obtained from shotgun sequencing data (**Figure 2d1, d2**). This led to a mutual control and confirmation of data (sequence alignment versus fingerprinting). The entire insert of BAC 7 (~22,500 bp) revealed a contig of 14,993 bp, separated from a second contig of 3,603 bp by a fragment of ~3,900 bp, flanked on either side by G+C-rich sequences of 349 and 776 bp, respectively (**Figure 2d**). The internal part of this fragment could not be sequenced completely. Subclones of this fragment revealed mainly the motif AGGGAG and degenerated versions of it, but no new sequences. Tetrameres of the AGGGAG motif and its complementary sequence were used as primers for PCR with only nucleotids and *Taq* polymerase. The primer-multimer products yielded a smear on agarose gels and strong FISH signals exclusively at all centromeres of barley (**Figure 3**) but not on rye and wheat centromeres.

From sequence comparison of BAC 7 with the components of *cereba* of λ9 a high degree of similarity became evident in spite of some rearrangements outside the polygene region (**Table 2** and **Figure 2c**). Within both contigs the RNA binding domains of the BAC 7 *cereba* elements show insertions of 119 bp (position 1640-1759) and 110 bp (position 9460-9570 and position 2349-2459 within the shorter contig, respectively) in addition to insertions within the 5‘ and 3‘ untranslated regions between the LTRs and the polygene regions as compared to the corresponding sequence of *cereba* of λ9 (**Table 2** and **Figure 2c**).

GISH with genomic DNA of barley and an excess (10-fold) of unlabelled BAC 7 DNA revealed strong signals along chromosome arms and very few signals at centromeric regions (**Figure 4**). This indicates that *cereba* and the AGGGAG satellite represent the major sequence components of all barley centromeres.

Table 1: Fragments of BAC 7 after complete digestions with 10 restriction enzymes and 20 pairwise combinations of these. Restriction fragments of BAC 7 were identified by Southern blot analyses with the vector, subclones of *cereba* representing the polygenic region of the retrotransposon (pGP 5, 7, 12, 13), and the supposed LTR (pGP 33).

Bgl II	**BstXI**	**EcoRI**	**HindIII**	**Kpn I**	**Not I**	**Pst I**	**Sal I**	**Sfu I**	**Xba I**
6.2	8.0	*9.2* 12 33 v	**7.5 V**	9.4	*~23.0*	~11.5 5 7 12 13 33	*~22.0* 5 7 12 13 33 v	*~14.0*	~11.3
4.4	*5.6*	8.0 5 7 12 13 33	4.8 *	*6.0*	**6.9**	4.3 12 33	**6.4 V**	7.0	8.0
3.9	*4.8*	**6.6 V**	4.1d 5 7 13	5.6		3.8 5 7 12 13	**0.82 V**	4.5	**5.3**
3.5 d	4.3	*5.0* 5 7 12 13 33 v	2.9 t 12 33	**3.27**		*3.4* 12 v		2.4	*2.7*
2.4	3.5 d	0.87 7	0.9 *	2.15 d		**2.9 V**		1.3	2.4
2.0				*1.15*		2.5 12 33		0.87	
1.5 d						**1.5 V**			
0.3 d									
Σ 29.5	29.7	29.67	30.1	29.72	29.9	29.9	29.22	30.07	29.7

Bgl II HindIII	**BstXI EcoRI**	**BstXI HindIII**	**BstXI Pst I**	**BstXI Sfu I**	**BstXI Xba I**	**EcoRI HindIII**	**EcoRI Not I**	**EcoRI Pst I**	**EcoRI Sal I**
4.8	8.0	4.8	8.2	*5.8*	8.1	**6.6 V**	8.0 d 5 7 12 13 33	8.4 12 13	*9.2* 12 33 v
3.3	4.3	**4.0**	3.8	*4.8*	4.3	4.8 *	**6.4 V**	4.3 12 33	8.0 5 7 12 13 33
2.3	**3.4**	**3.4**	*3.4*	4.6	**3.4** t	2.9 t 5 7 12 33	8.0 d 5 7 12 13 33	**2.9 V**	**5.8 V**
2.1 t	**3.2**	2.8 d	3.2 d	3.5 d	*2.6*	2.0 d 5 7 13	0.87 7	*2.5* 12 33 v	5.0 5 7 12 13 33
2.0	2.7 d	2.3	**2.9**	2.4 d	2.2	1.9 5 7 13	**0.6 V**	2.4 d 5 7 12 13	0.87 7
1.4 q	2.3	2.2 d	2.3	1.1	**1.9**	1.2 5		**2.2 V**	**0.8 V**
0.9 t	*1.6*	1.3 d	**1.0**	0.95		0.9 *		**1.5 V**	**0.56 V**
0.68 d	0.8 d	0.9	**0.5** d			0.87 7		*1.2* d 5 7 12 v	
0.4		0.7 t				**0.8 V**		0.87 7	
0.3									
Σ 29.06	29.8	29.4	29.0	29.05	29.3	29.77	28.87	29.87	30.23

EcoRI Sfu I	**EcoRI Xba I**	**HindIII Not I**	**HindIII Pst I**	**HindIII Sfu I**	**HindIII Xba I**	**Not I Pst I**	**Pst I Sfu I**	**Pst I Xba I**	**Sfu I Xba I**
6.6	8.3	**6.9 V**	4.8 *	**7.5**	**5.3**	~11.3 5 7 12 13 33	4.8	~ 11.3	**5.3** d
5.2	**5.3** d	4.8 *	4.1 5 7 13	4.3	4.8	4.3 12 33	4.5	4.3	4.6
5.0	2.5 d	4.1 d 5 7 13	3.3 5 7 13	3.8 d	4.1 d	3.8 5 7 12 13	3.8	3.8	4.2 d
4.5	2.4	2.9 t 12 33	**3.0 V**	2.7 t	2.4 t	**2.7 V**	*3.4*	**2.9**	*2.7*
4.2	**1.3** d	0.9 *	**2.9 V**	0.9 d	**2.2**	**2.6 V**	**2.9**	*2.6*	2.6
2.4	0.87	0.4 * (?)	2.5 t 12 33		0.9	2.5 12 33 v	2.7 d	2.4	2.3
1.8			**1.5 V**		0.5 d	**1.5 V**	2.5	**1.5**	
0.9			0.9 *			*0.6* 12 v	**1.5**	**0.8**	
0.87			0.78 *			*0.32* * (?)	0.85		
			0.4 t 12						
Σ 31.47	29.77	29.9	29.98	29.3	29.6	29.58	29.65	29.6	31.2

numbers - sizes of a fragments in kb;

bold - fragments representing only sequence of the vector pBeloBAC 11 (**V**);

italics - fragments composed of vector (v) and insert sequences; where Southern blot was not performed, the fragments corresponding either to the vector, or to the vector and insert sequences were determined by comparison with restriction fragments known for the vector;

* fragments that hybridized neither with sequences of *cereba*, nor with the vector;

d, t, q - double, triple, quadruple band;

(?) fragments, which should hybridize with the vector, but did not.

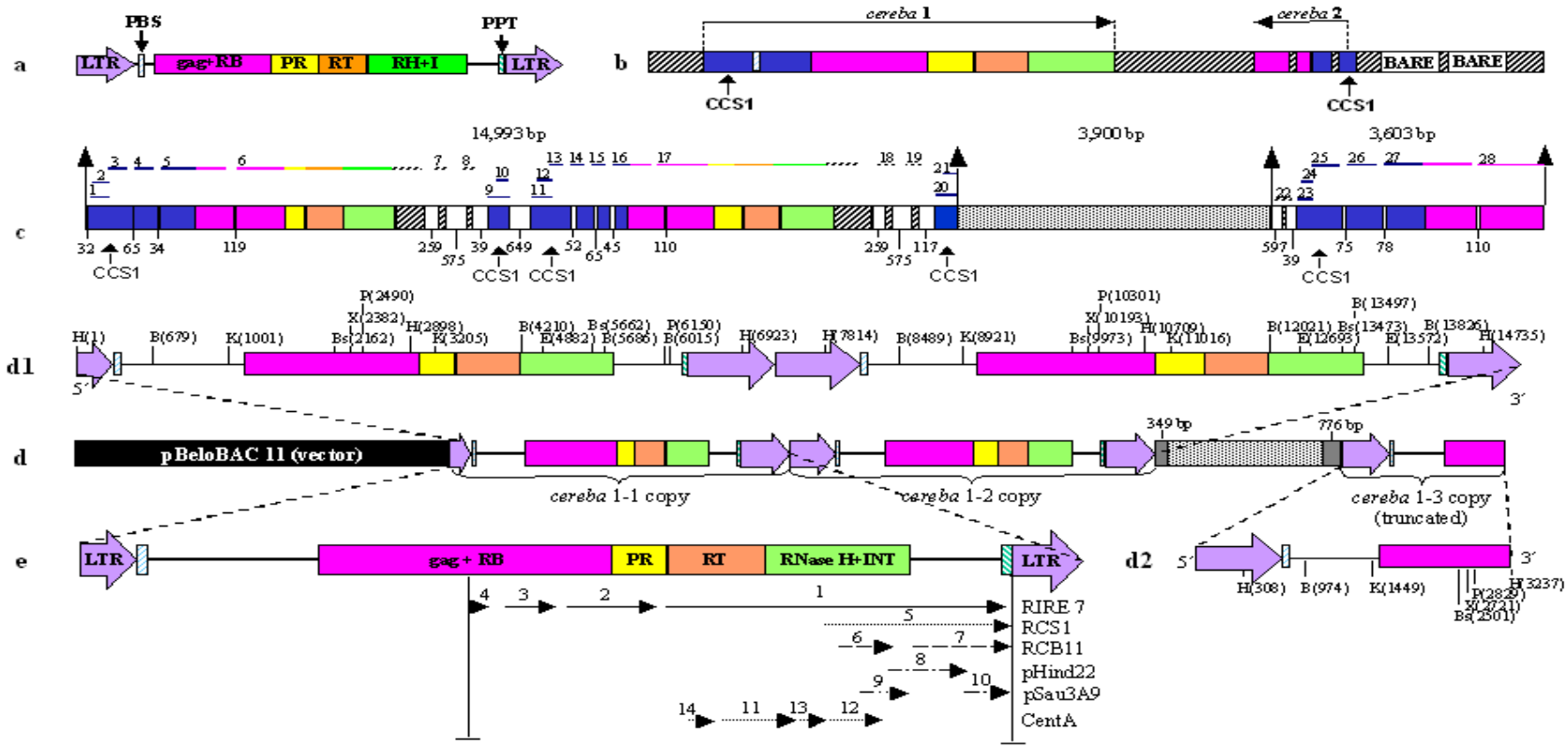

Figure 2: Sequence organization of the barley clone BAC 7. **(a)** Scheme of a *gypsy*-type retrotransposon; **(b)** Scheme of the insert of λ9 clone (15,230 bp); **(c)** Organization of the insert of BAC 7 in comparison with λ9 (see Tab. 2); **(d)** The entire clone BAC 7; **(d1)** Restriction map of the contig of 14,993 bp of the BAC 7 insert; **(d2)** Restriction map of the contig of 3,603 bp; **(e)** Scheme of the first retrotransposon copy of the contig of 14,993 bp in comparison with other plant sequences (see Tab. 3). violet arrows: position and orientation of the LTRs; **black** bars: 3′ and 5′ untranslated regions; dark blue: homologous to parts of subclone of *cereba* (pG33) including the CCS1 domain; gag+RB: gag gene (encoding structural proteins involved in intracellular packaging of the RNA transcript) + RNA binding site; PR: protease; RT: reverse transcriptase; RH+I: RNase H + integrase; **BARE**: sequences containing regions of homology to a *copia*-like element of barley (Manninen and Schulman 1992) and to related dispersed repeat elements of rye (Rogowsky *et al.* 1992); **PBS**: primer binding site, hatched in blue; **PPT**: polypurine tract, hatched in green; dotted: non-sequenced region (~3.9 kb); gray: G+C-rich domains of BAC 7; hatched in black: regions corresponding to insert of λ9 outside of *cereba* sequences; white: sequence absent from λ9, numbers below (c) indicate length in bp; arrows in (b): orientation of the polygene domain; coloured bars in (c) specify regions of BAC 7 aligned with λ9 (see Table 2); numbers in (e) correspond to sequences specified in Table 3; numbers in parentheses next to enzymes (B=*Bgl* II, Bs=*Bst* XI, E=*EcoR* I, H=*Hind* III, K=*Kpn* I, P=*Pst* I, X=*Xba* I) indicate the positions of restriction sites in (d1) and (d2).

Table 2: Sequence comparison (BLASTN) of BAC 7 and λ9 (compare Fig. 2c)

n	contig 1 (14,993 bp) (positions)	λ 9 (positions)	similarity (%)	identity (in bp)
1	33-262	2038-1815	80	187/231
2	104-262	11643-11489	83	132/159
3	253-833	2074-2645	84	496/589
4	898-973	2705-2780	89	68/76
5	1007-1639	2826-3464	91	582/639
6	1760-5980	3561-7777	94	3976/4227
7	6239-6277	8556-8517	90	36/40
8	6852-6896	7835-7791	88	40/45
9	6935-7176	2055-1820	81	198/243
10	7023-7160	11643-11508	83	115/138
11	7826-8071	2055-1815	81	201/247
12	7914-8071	11643-11489	82	131/158
13	8062-8536	2074-2542	85	409/481
14	8588-8642	2591-2645	92	51/55
15	8707-8782	2705-2780	89	68/76
16	8827-9459	2826-3464	90	581/693
17	9571-13791	3561-7777	94	3976/4227
18	14050-14088	8556-8517	90	36/40
19	14663-14707	7835-7791	88	40/45
20	14824-14977	1984-1834	81	122/155
21	14843-14977	11640-11508	78	110/135
	contig 2 (3,603 bp)			
22	598-642	7835-7791	88	40/45
23	681-925	2055-1815	80	199/246
24	769-925	11643-11489	81	128/157
25	916-1488	2074-2645	85	496/581
26	1563-1638	2705-2780	88	67/76
27	1716-2348	2826-3464	90	580/639
28	2460-3603	3561-4698	90	1041/1144

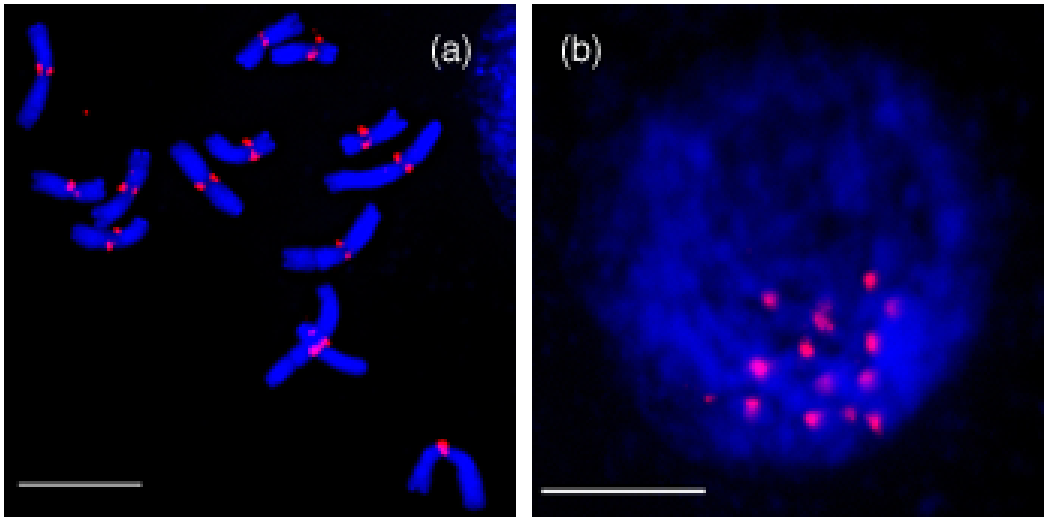

Figure 3: FISH with $(AGGGAG)_n$, amplified and biotin-labelled by PCR, yielded signals on all centromeres of metaphase chromosomes **(a)** and interphase nuclei **(b)** of barley karyotype MK14/2034. Bars = 10 µm

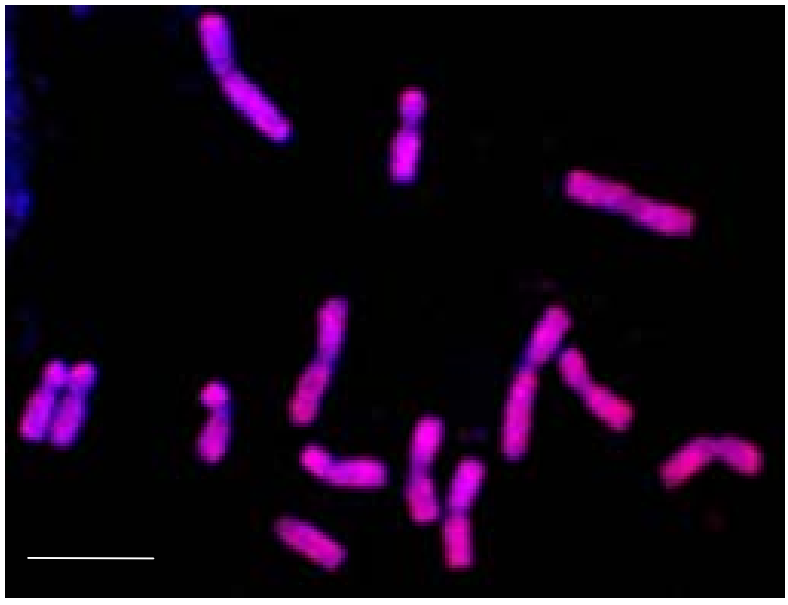

Figure 4: GISH with genomic barley DNA biotin-labelled, and an excess (10-fold) of unlabelled BAC 7 DNA yielded strong signals along chromosome arms and very few signals at centromeric regions of barley karyotype MK14/2034. Bar = 10 µm

3.1.3 The contig of 14,993 bp contains two *cereba* elements in tandem

The left contig of the BAC 7 insert is formed by two almost identical and complete *Ty3*/*gypsy*-like retroelements of high similarity to *cereba* of λ9. Both contained all five catalytic regions (RNA binding site/protease/reverse transcriptase/RNase H/integrase) including *gag*, primer binding sites and polypurine tracts and are flanked by LTRs on both sides. The upstream LTR of the first element (position 1-257) lacks the first 665 bp. The primer binding site (PBS) follows immediately at its 3′ end (position 258-274). The downstream LTR (922 bp) with a terminal TGAT/ATCA inverted repeat is preceded by a polypurine tract (PPT) at position 6242-6254 (**Figure 2d1**). The second copy of the *cereba* element is complete except for the first 23 bp lacking at the upstream LTR. The 5′ regions of the (almost) complete LTRs show similarity (~50%) with the LTR sequence of the RIRE 7 *gypsy*-type retrotransposon in rice (Kumekawa *et al.* 2001) and the last third of LTRs (260 bp) with the CCS1 (Aragón-Alcaide *et al.* 1996) sequence (80%).

Both retrotransposons show extended homology to the sequence RCB 11 (AB013613, see Nonomura and Kurata 1999), the *gypsy*-type retrotransposon RIRE 7 (AB033235, see Kumekawa *et al.* 2001) and the repeat RCS1 (AF078903, see Miller *et al.* 1998a; Dong *et al.* 1998) of rice, to the repeats *pSau3A9* (SBU68165, see Jiang *et al.* 1996) and *pHind22* (AF078901, see Miller *et al.* 1998a) of sorghum, as well as to the retrotransposon–like repeat CentA of maize (AF078917, see Ananiev *et al.* 1998), which all occupy centromeric positions (see **Table 3**, **Figure 2e**).

Table 3: Comparison (BLASTN) of the first retrotransposon copy on contig of 14,993 bp (position 1-7176) of BAC 7 with other known plant centromeric sequences. Numbers in parentheses indicate the position of sequences shown in Figure 2e.

	similarity to domain /accession/	position on the original accession	position on BAC 7	identity (%)
O. sativa	polyprotein region of the *gypsy*-like retrotransposon RIRE 7 /AB033235/; (Kumekawa *et al.* 2001)	3187-5896	3545-6254 [1], 11356-14065	81
		2579-3375	2943-3375 [2], 10754-11186	72
		1884-2091	2248-2455 [3], 10059-10266	73
		1608-1662	1972-2026 [4], 9783-9837	81
O. sativa subsp. *indica*	dispersed centromeric repeat family RCS1 /AF078903/; (Miller *et al.* 1998a, Dong *et al.* 1998)	27-1184	5119-6275 [5], 12930-14086	82
O. sativa	centromeric sequence RCB 11 /AB013613/; (Nonomura and Kurata 1999)	2227-2681	5128-5582 [6], 12939-13393	81
		3121-3372	6022-6273 [7], 13833-14084	83
S. bicolor	centromeric sequence pHind22 /AF078901/; (Miller *et al.* 1998a)	20-366	5647-5993 [8], 13458-13804	82
S. bicolor	centromeric sequence pSau3A9 /SBU68165/; (Jiang *et al.* 1996)	5-324	4996-5315 [9], 12807-13126	82
		338-422	6184-6268 [10], 13995-14079	90
Z. mays	centromeric retrotransposon-like repeat CentA /AF078917/; (Ananiev *et al.* 1998)	698-1071	3982-4355 [11], 11793-12166	79
		6162-6402	4810-5050 [12], 12621-12861	82
		5949-6094	4597-4742 [13], 12408-12553	82
		407-492	3691-3776 [14], 11502-11587	86

3.1.4 The contig of 3,603 bp contains a 3'-truncated cereba element

The right contig of the BAC 7 insert between the G+C-rich stretch and the vector covers a *cereba* element extending from the 5' LTR (position 1-920) with the terminal TGAT/ATCA inverted repeat up to the end of the *gag*+RB region. This element is nearly identical with the corresponding parts of the complete *cereba* elements of the left contig.

3.1.5 Conclusions as to the sequence composition and sequence organization of barley centromeric DNA

i. The insert of BAC 7 (~23 kb), was found to harbour three copies of the *Ty3*/*gypsy*-like retroelement *cereba* (two complete and one truncated) flanked by LTRs of nearly 1 kb, and a sequence with the predominant motif AGGGAG. Both sequences are centromere-specific for barley. The *cereba* elements and the G+C-rich satellite sequences compose the major DNA component of all barley centromeres. The CCS1 sequences are indeed parts of LTRs, as proposed by Presting *et al.* (1998). While the *cereba* element shows high similarity to *gypsy*-like elements within centromeres of other cereals, the G+C-rich satellite is barley-specific. Similar data about centromeric DNA composition including *gypsy*-type retroelements and species-specific short tandem repeats were recently reported for other cereals (Kumekawa *et al.* 2001; Nonomura and Kurata 2001; Cheng *et al.* 2002).

ii. The results of sequence and restriction analysis of BAC 7 are in accordance with the previous assumption that the λ9 insert is not representative for the sequence organization within barley centromeres. Similar clones, combining centromeric *gypsy*-like and non-centromeric *copia*-like elements, were also reported for sorghum (Miller *et al.* 1998a) and maize (Ananiev *et al.* 1998). These clones are either chimeric or originate from centromere-flanking regions.

iii. It might be possible that centromere-specific satellites such as the G+C-rich sequence motif of barley have originated during evolution by nested transposition (SanMiguel *et al.* 1998); their redundancy may depend on species- and position-specific transposition frequencies of certain types of mobile elements which may

(Langdon *et al.* 2000) or may not be identical with those found to be clustered at cereal centromeres.

iv. About 200 *cereba* elements of ~7 kb each, are present per barley centromere (Presting *et al.* 1998). This indicates a considerably higher density than calculated for wheat (one *gypsy*-like element per 55 kb; Fukui *et al.* 2001) or sorghum centromeres (two such elements within 90 kb; Miller *et al*. 1998a). Also the completeness of the *cereba* elements is a novelty when compared to that within centromeric clones of other cereals (see Langdon *et al.* 2000).

v. It is suggested, that *gypsy*-type of retroelements such as *cereba* do not frequently invade non-centromeric positions within their host genomes. Apparently, they are conserved within the centromeres of all cereals since their radiation ~60 Myr ago, due to vertical transmission (Kumar and Bennetzen 1999).

vi. The functional meaning of *gypsy*-like retroelements within cereal centromeres is not yet clear. Although their number may be reduced below the detectability by FISH within mitotically and meiotically stable barley telosomic (T. R. Endo, pers. communication), they are apparently involved in recruiting CENP-A like kinetochore proteins in maize (Zhong *et al.* in press). These results leave open the question whether or not kinetochore assembly at cereal centromeres is epigenetically regulated.

vii. Centromeric sequences from barley are now available to test for interactions with suitable (constitutive) plant kinetochore proteins by gel shift, plasmon resonance or chromatin immunoprecipitation assays.

3.2 Impact of recombinantly elongated chromosome arms on nuclear divisions and plant development

3.2.1 Occurrence, phenotype and fertility of barley cytotypes with recombinantly elongated chromosome arms

Barley chromosome 7, with a satellite on its short arm, is the longest chromosome of the normal karyotype (W). It comprises 14.9% of the total metaphase genome length. The long arm of chromosome 7 represents the longest arm of W (9.0% of the metaphase genome length corresponding ~5.8 μm; **Figure 5a**). To study the upper tolerance limit for chromosome arm length in a monocotyledonous plant, F_2 individuals derived from five crosses, each between two barley translocation lines were investigated. The parental translocation lines for each cross were selected in a way that one chromosome was involved in both translocations, exchanging unequal parts of the chromosome arms involved. In these cases crossing-over between the homologous regions of the translocation chromosomes in the five doubly heterozygous F_1 progenies were expected to generate recombinantly elongated chromosome arms comprising 12.3%, 13.4%, 14.0%, 14.7% and 15.1% of the metaphase genome length, respectively (**Figure 6**). At least for the latter three cases, the arm length might occasionally exceed half of the spindle axis extension during mitotic telophase in barley (see below). However, the expected meiotic recombination event occurred only in one of the five crosses (T1-6y × T1-7f) although several hundred F_2 individuals of each cross combination were inspected (**Table 4**). For identification of chromosomes with recombinantly elongated chromosome arms in F_2 progenies from the crosses T2-4aw × T2-3am and T1-5a × T5-7ac, in addition to Giemsa N-banding, fluorescent in situ hybridization with the subtelomeric repeat HvT01 was applied, since the corresponding arms should reveal

either a double signal, characteristic for the terminus of the arm 3L, or a very weak signal typical for the terminus of the arm 7L (**Figure 7**; Schubert *et al*. 1998b). The reason for the lack of recombinants with elongated chromosome arm from four crosses is most likely that the corresponding chromosome regions are only rarely involved in recombination, while the region relevant for the cross T1-6y × T1-7f represents a recombination hot spot (see **Figure 6** and Künzel *et al*. 2000).

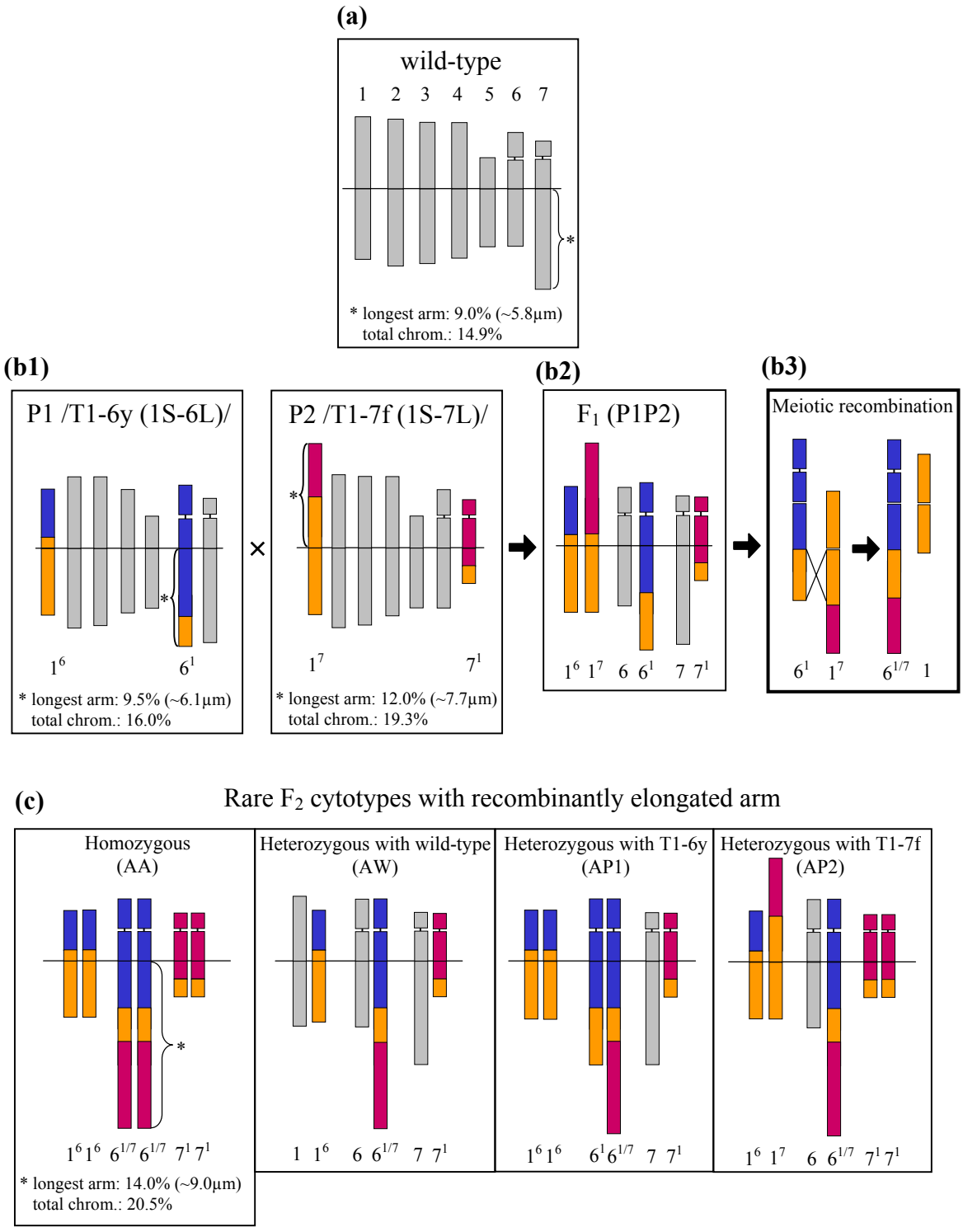

Figure 5: Idiograms of *Hordeum vulgare* wild-type **(a)**, crossed translocation parental lines T1-6y (P1) and T1-7f (P2) **(b1)** and the double heterozygote P1P2 (F_1) **(b2)**. Crossing-over in F_1 individuals between the homologous regions of the translocation chromosomes 6^1 and 1^7 **(b3)** and karyotypes with recombinantly elongated long arm of chromosome $6^{1/7}$ **(c)**.

Designation of chromosomes according to the old nomenclature for barley chromosomes.

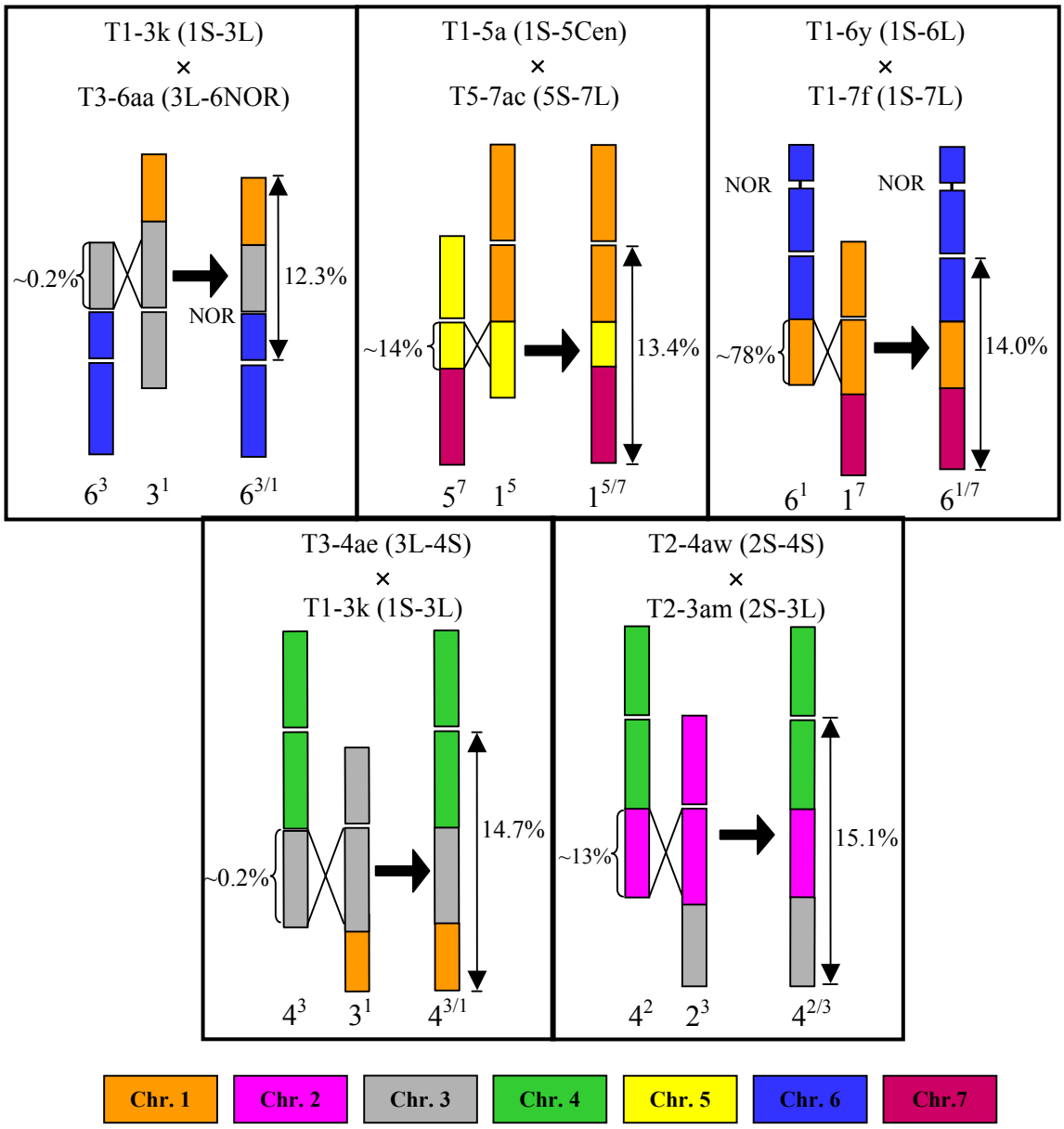

Figure 6: Scheme of chromosome arm elongation expected by meiotic recombination between translocated chromosomes in heterozygous F_1 individuals resulting from 5 pairwise crosses of translocation lines of barley. Recombinative chromosome arm elongation was found only within the F_2 progeny of cross T1-6y × T1-7f. The reason is that the proportion of the average recombination frequency of the homologous regions as compared to the entire wild-type arm is too low (0.2; 14; 0.2 and 13%) except for that of chromosomes $6^1/1^7$ (78%). For description of translocation lines see http://wheat.pw.usda.gov/ggpages/Barley_physical/Idiograms/.

NOR = nucleolar organizer

Table 4: Number of F_2 individuals with recombinantly elongated chromosome arms from 5 crosses between selected translocation lines (see Figure 6).

Cross	Length of longest arm (%**/µm)	Length of spindle in telophase required for complete separation (µm)	No. of F_2 individuals tested	Individuals with elongated arm
T1-3k × T3-6aa*	12.3/8.0	16.0	371	-
T1-5a × T5-7ac *	13.4/8.6	17.2	682	-
T1-6y × T1-7f	14.0/9.0	18.0	918	39
T1-3k × T3-4ae *	14.7/9.5	19.0	538	-
T2-4aw × T2-3am *	15.1/9.8	19.5	750	-

* for karyotype description see http://wheat.pw.usda.gov/ggpages/Barley_physical/Idiograms/

** % of metaphase genome length

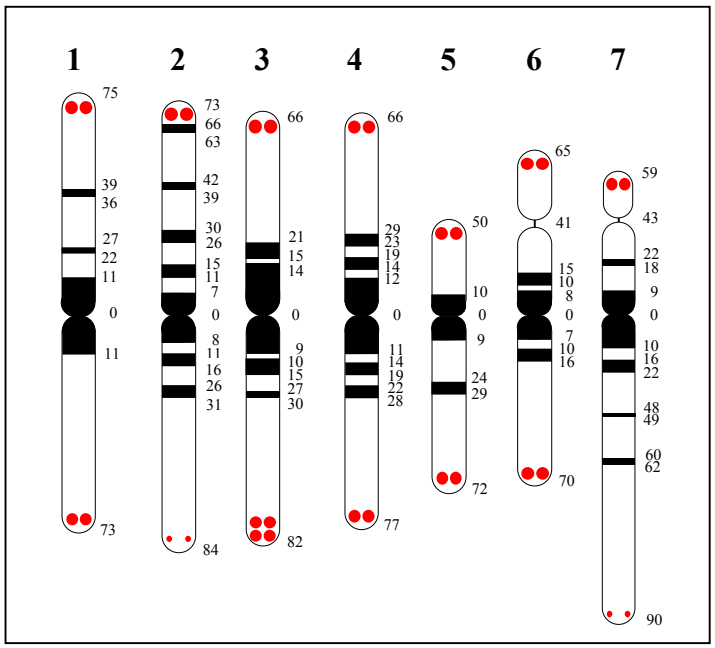

Figure 7: Scheme of *Hordeum vulgare* standard N-banded chromosomes and FISH signals (red dots) using subtelomeric repeat (HvT01; Schubert *et al*. 1998b) as probe. All five crosses were inspected using Giemsa N-banding technique and FISH with the subtelomeric repeat HvT01 was applied in crosses T2-4aw × T2-3am and T1-5a × T5-7ac (see Fig. 6).

numbers indicate distances in milligenome units

From self-pollinated F_1 plants, of the cross T1-6y × T1-7f (**Figure 5b**), F_2 seedlings with elongated chromosome arms were obtained indicating meiotic recombination between homologous regions of the parental translocation chromosomes 6^1 and 1^7. The recombinantly elongated chromosome ($6^{1/7}$) of the new recombinant karyotype (A) covers 20.5% and its longer arm 14.0% (~9.0 μm) of the total metaphase genome length (**Figure 5c**). Gametes containing either the elongated chromosome $6^{1/7}$ or wild-type chromosome 1, the reciprocal recombination product, occurred with similar frequencies. Among 921 F_2 individuals, 39 were found to have a karyotype resulting from recombinant chromosome elongation; 32 of these were heterozygotes with one of the parental translocation karyotypes (AP1 or AP2) (**Figure 8a**). One was a homozygote (AA) (**Figure 8b**), two were heterozygotes with the normal karyotype (AW) (both contained chromosome 6^1 as a tertiary trisomic) and four contained chromosome $6^{1/7}$ as a tertiary trisomic in P2P2, P1P2 or WP2 background (**Table 5a**). The remaining individuals possessed the karyotypes P1P1 (202), P2P2 (202), P1P2 (452), WP1 or WP2 (26). In total, among 1842 gametes 36 were of recombination karyotype A and 29 of the reciprocal normal karyotype. This means that ~3.5% of the gametes had karyotypes that arose from recombination between chromosomes 6^1 and 1^7.

Among the selfed progeny of AP1 plants, the ratio of karyotypes AA:AP1:P1P1 was not significantly different (P = 0.76) from the expected Mendelian ratio (1:2:1) (**Table 5b**). Self-pollination of the AW plants with chromosome 6^1 as a tertiary trisomic yielded 18 plants with balanced karyotypes (three of AA, nine of AP1, four of P1P1 and two of WP2 karyotype) which involved 15 gametes of karyotype A, 19 of karyotype P1 and two of the wild-type (**Table 5c**).

Except for reduced fertility, the F_1 (P1P2; 21 grains/100 spikelets) and the heterozygous AP1 plants (42 grains/100 spikelets) were phenotypically indistinguishable from homozygous P1P1, P2P2 and wild-type plants. The plants homozygous for the

elongated chromosome arm (AA) were slower growing and less vigorous than wild-type plants (**Figure 9**). Compared to the wild-type (93 grains/100 spikelets) and the homozygous parent P1P1 (77 grains/100 spikelets) the fertility of homozygous AA plants (34 grains/100 spikelets) was significantly reduced according to the Student-Newman-Keuls test ($P < 0.05$), comparable to that of the heterozygous populations P1P2 and AP1. The amount of spikelets per spike between all karyotypes tested was not significantly different ($P = 0.397$).

Table 5: Karyotypes with recombinantly elongated long arm of chromosome $6^{1/7}$ among 921 F_2 individuals of the cross T1-6y × T1-7f **(a)**, among 68 F_3 individuals after selfing of AP1 **(b)** and among 18 F_3 individuals after selfing of AW (2n=15) **(c)** (see Figure 5c).

a) Karyotypes with elongated chromosome arm observed in F_2		Number of individuals
heterozygous with T1-6y (AP1)	$1^6 1^6\ 6^1 6^{1/7}\ 7^1 7^1$	14
heterozygous with T1-7f (AP2)	$1^6 1^7\ 6 6^{1/7}\ 7^1 7^1$	18
homozygous (AA)	$1^6 1^6\ 6^{1/7} 6^{1/7}\ 7^1 7^1$	1
heterozygous with wild-type (AW)	$1\ 1^6\ 6 6^{1/7}\ 7 7^1 + 6^1$ (2n=15)	2
homozygous T1-7f (P2P2)	$1^7 1^7\ 6 6\ \ 7^1 7^1 + 6^{1/7}$ (2n=15)	2
T1-6y / T1-7f (P1P2)	$1^6 1^7\ 6 6^1\ 7 7^1 + 6^{1/7}$ (2n=15)	1
wild-type heterozygous with T1-7f (WP2)	$1 1^7\ 6 6\ \ 7 7^1 + 6^{1/7}$ (2n=15)	1

b) Karyotypes observed in F_3 after selfing F_2 individuals heterozygous with T1-6y (AP1)		Individuals expected (%)	Individuals observed
homozygous (AA)	$1^6 1^6\ 6^{1/7} 6^{1/7}\ 7^1 7^1$	25	18
heterozygous with T1-6y (AP1)	$1^6 1^6\ 6^1 6^{1/7}\ 7 7^1$	50	31
homozygous T1-7f (P1P1)	$1^7 1^7\ 6 6\ \ 7^1 7^1$	25	19

c) Karyotypes observed in F_3 after selfing F_2 individuals heterozygous with wild-type (AW) (2n=15)		Number of individuals
homozygous (AA)	$1^6 1^6\ 6^{1/7} 6^{1/7}\ 7^1 7^1$	3
heterozygous with T1-6y (AP1)	$1^6 1^6\ 6^1 6^{1/7}\ 7 7^1$	9
homozygous T1-7f (P1P1)	$1^7 1^7\ 6 6\ \ 7^1 7^1$	4
wild-type heterozygous with T1-6y (WP1)	$1 1^6\ 6 6^1\ 7 7$	2

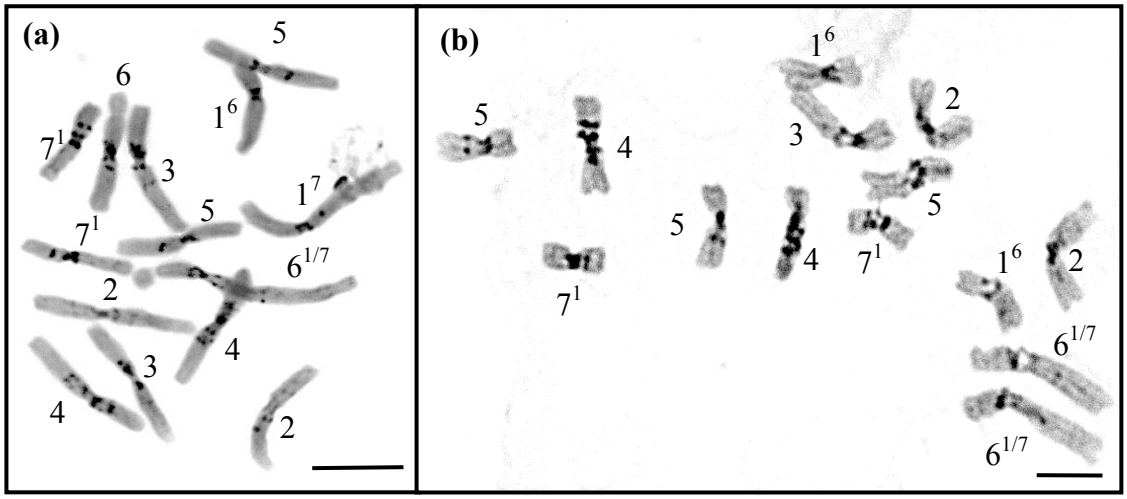

Figure 8: Giemsa N-banded somatic metaphase cells of *Hordeum vulgare* heterozygous with parent T1-7f **(a)** and homozygous **(b)** for chromosome arm elongation ($6^{1/7}$) by meiotic recombination between chromosome 6^{1} and 1^{7} after crossing of T1-6y × T1-7f. Bars = 5 µm.

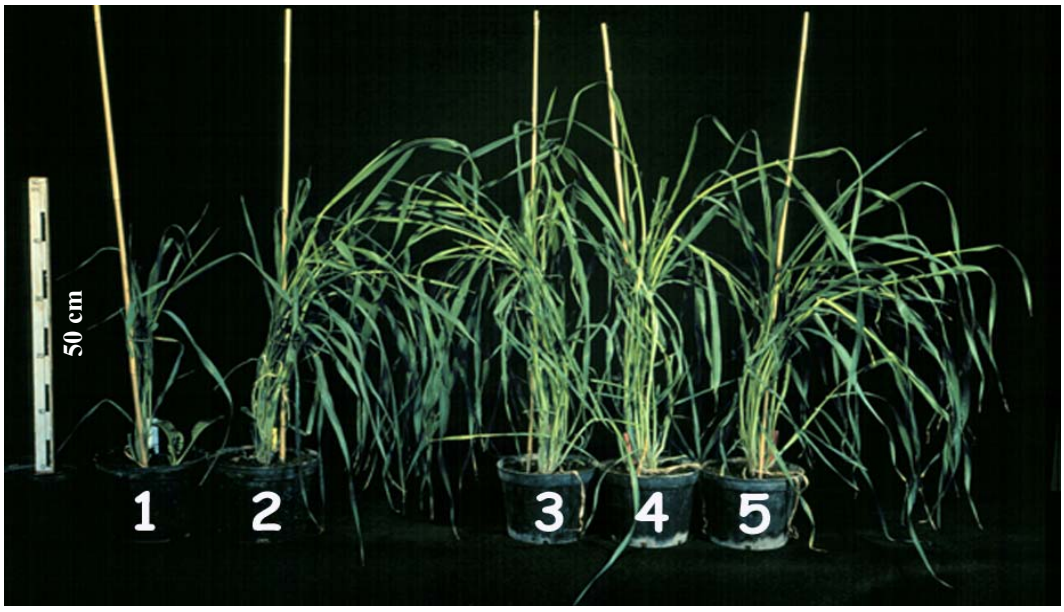

Figure 9: Phenotype of *Hordeum vulgare* plants with homozygously (AA) **(1)** and heterozygously (AP1) **(2)** elongated chromosome $6^{1/7}$. Plants **(3)**, **(4)** and **(5)** are of P1P1, P1P2 and wild-type karyotype, respectively. All plants are of the same age.

3.2.2 Mitotic spindle axis length, separation of sister chromatids and formation of micronuclei in karyotypes with elongated chromosome arms

To make comparative studies on the spindle length, its extension from anaphase to telophase and on mitotic sister-chromatid separation in karyotypes differing in the length of their longest chromosome arm, plants of the normal karyotype, of the karyotype MK 14/2034 and of karyotypes possessing chromosome $6^{1/7}$L (AA, AP1, AP2 individuals) were studied. The length of the longest arm was 5.8, 7.8 and 9.0 µm, respectively (**Figure 10**, **Table 6**). The average anaphase spindle axis extension in root meristem cells was similar (14.2-15.1 µm) for all karyotypes. Theoretically, the minimum spindle length required for complete sister-chromatid separation at telophase should be 11.6 µm for the normal karyotype, 15.6 µm for MK 14/2034 and 18.0 µm for the A karyotype (twice the longest arm length, see **Fig. 10**). In fact, the average telophase spindle length was 16.8 µm for the normal karyotype, 16.3 µm for MK14/2034 and 17.7 µm for A (**Table 6**). This means, that the longest arms of the normal karyotype and of karyotype MK 14/2034 can be separated regularly during telophase, while for the long arm of chromosome $6^{1/7}$ an incomplete separation of sister-chromatids might be expected for cells with an average or shorter length of the spindle axis (**Fig. 10**). The maximum length of the telophase spindle (27-28 µm) was similar for the three karyotypes studied (**Table 6**).

Mitotic and post-mitotic cells of root meristems of wild-type, AP1, AP2 and AA plants were inspected as to the occurrence of non-separated sister-chromatids and the formation of micronuclei. All sister-chromatids were completely separated in all dividing cells examined in the normal karyotype, whereas overlapping of sister-chromatid termini of the longest arm was observed at telophase in 25 out of 106 AP1 and AP2 cells and in 30 out of 100 cells of karyotype AA (**Table 7**, **Fig. 10 c2**). As a consequence, a micronucleus was found in 2.5% of AP1 and AP2 cells and in 2.7% of

AA cells (**Table 7**, **Figure 10 c3**), while no micronuclei were detectable in cells of the normal karyotype.

Apparently, non-separation of sister chromatids in karyotypes carrying chromosome $6^{1/7}$ did not significantly influence the duration of telophase, because the proportion of telophase cells in these karyotypes was similar to that of wild-type plants (**Table 7**).

Table 6: The length of the longest chromosome arm and spindle axis extension in *Hordeum vulgare* root meristem cells of wild-type (W), of the translocation line MK14/2034 and of karyotypes (AA; AP1; AP2) with the arm $6^{1/7}$L elongated by 56% as compared to the longest arm of the wild-type.

Karyotype	Longest arm late A (% */μm)	Number of cells		Spindle axis extension (μm)		
		late A	T	late A ± SD	T ± SD	T max.
W	9.0/5.8	50	50	14.2 ± 1.57	16.8 ± 1.78	27.0
MK14/2034	12.1/7.8	20	20	15.1 ± 2.42	16.3 ± 3.37	28.0
AA; AP1; AP2	14.0/9.0	330	490	14.6 ± 2.14	17.7 ± 2.18	27.0

A – anaphase; T – telophase; * % of metaphase genome length

Table 7: Proportion of mitotic stages; Occurrence of incompletely separated chromosome arms; Proportion of cells with a micronuclei in root meristems of *Hordeum vulgare* wild-type (W), and karyotypes with heterozygously (AP1; AP2) and homozygously (AA) elongated long arm of chromosome $6^{1/7}$.

Karyotype	Counted cells at mitosis							Number of cell at late A/T with			No. of cells with MN	
	P	M	A	T	I	Total	Mitotic index	all arms separated (%)		longest arm not separated	0	1
W	38	15	10	10	1080	1153	6.3	100	(100)	0	1000	0
AP1; AP2	57	23	17	10	1050	1157	9.2	81	(76)	25	975	25
AA	40	16	17	10	930	1013	8.2	70	(70)	30	973	27

P – prophase; M – metaphase; A – anaphase; T – telophase; I – interphase; MN - micronucleus

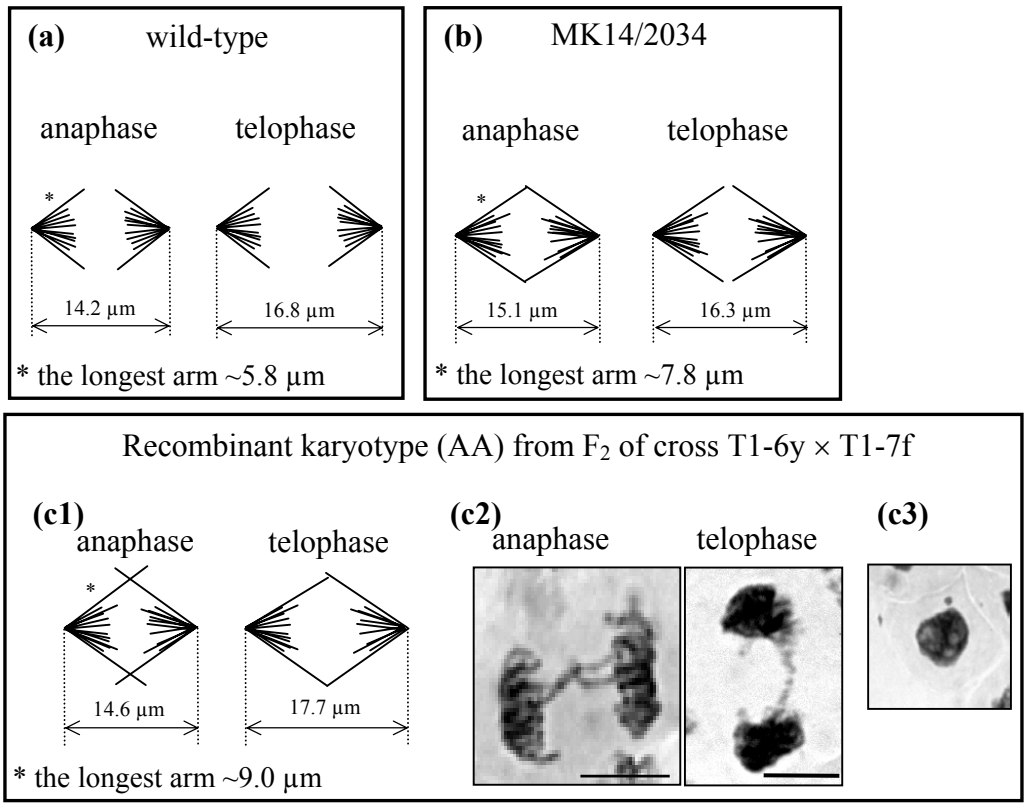

Figure 10: Scheme of late anaphase and telophase configurations of the wild-type **(a)**, plants of karyotype MK 14/2034 **(b)** and of the karyotype with recombinantly elongated arm of chromosome $6^{1/7}$ within F_2 progenies of the cross T1-6y × T1-7f **(c1)**. Mitotic cells of karyotype AA **(c2)**. Micronucleus in an interphase cell as a consequence of non-separated chromatids at telophase disrupted by the newly formed cell wall (micronucleus appeared in AP1, AP2 and AA karyotypes) **(c3)**. Bars = 10 µm.

3.2.3 Meiotic division and spindle axis length in karyotypes with elongated chromosome arms

To investigate the influence of the elongated arm of chromosome $6^{1/7}$ on meiotic chromosome separation, meiotic stages in wild-type, P1P1, AP1 and AA plants were compared and the spindle length during the second meiotic division of pollen mother cells as the pole-to-pole distance in late anaphase II cells was examined. Incomplete separation of elongated chromosome arms was not observed in any case (**Figure 11**). The spindle length at anaphase II was found to be 25.2, 22.4, 22.8 and 20.5 µm in wild-

type, P1P1, AP1 and AA plants, respectively (**Table 8**). This shows that the spindle is about 5-9 μm longer in meiotic than in mitotic cells. Since the dimensions of the meiocyte and spindle axis are similar during first and second meiotic divisions (**Fig. 11a**), complete separation of sister-chromatids of the elongated chromosome arm $6^{1/7}$L is easily possible during meiosis.

While the reduced fertility of P1P2 and AP1 plants seems to be caused mainly by lagging chromosomes from multivalents which form tetrads with 'additional' nuclei (**Fig. 11**, **Table 8**), the reduced fertility of the homozygous karyotype AA is likely due to mitotic disturbances during early embryonic divisions caused by incomplete separation of sister-chromatids of the chromosome arm $6^{1/7}$L.

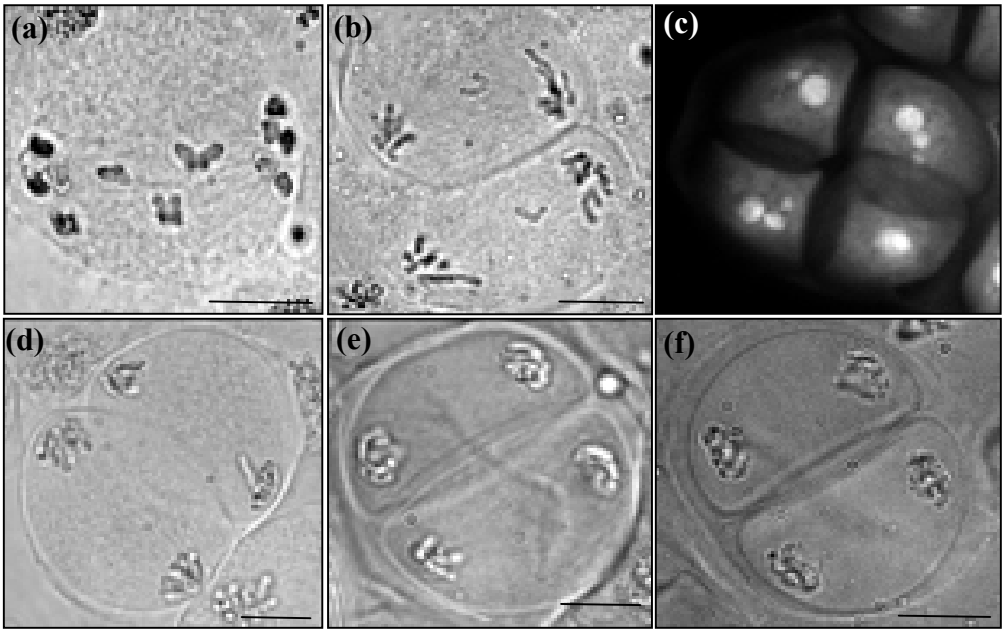

Figure 11: Meiotic disturbances (lagging chromosomes) in heterozygous AP1 plants occurring at anaphase I **(a)**, anaphase II **(b)** (phase contrast images) and formation of 'additional' nuclei from laggards in tetrads **(c)** (DAPI stained). Correct telophase II of wild-type **(d)**, P1P1 **(e)** and AA **(f)** plants (phase contrast images). Bars = 10 μm

Table 8: Spindle extension during 2nd meiotic division, lagging chromatids and tetrads with 'additional' nuclei (MN) in wild-type (W) plants, the homozygous parental translocation line P1P1 and plants with elongated long arm of chromosome $6^{1/7}$ in heterozygous (AP1) and homozygous (AA) condition.

Karyotype	Spindle length meiosis II [µm] ± SD (n)	Correct segregation ana- II, telo- II n (%)	Aberrant segregation * ana- II, telo- II n (%)	Tetrades * without MN n (%)	with MN
W	25.2 ± 2.48 (22)	130 (100)	0 (0)	200 (100)	0
P1P1	22.4 ± 2.14 (83)	148 (99)	2 (1)	200 (100)	0
AP1	22.8 ± 3.37 (41)	180 (90)	20 (10)	93 (93)	7
AA	20.5 ± 2.39 (50)	198 (99)	2 (1)	200 (100)	0

* disturbed segregation and 'additional' nuclei were due to lagging chromatids but not to incomplete separation of $6^{1/7}$L chromatids (see Fig. 11)

n – number of meiocytes

3.2.4 Conclusions as to the upper limit for chromosome arm length in barley

i. In barley, incomplete mitotic separation of sister chromatids of chromosome arms elongated experimentally by ~30%, the occurrence of micronuclei within ~3% of the meristematic cells and the reduced vigour of the recombinant cytotypes are in correspondence with data reported for *Vicia faba* (Schubert and Oud 1997).

ii. In both species, barley a monocot and *Vicia* a dicot, chromosome arms which only slightly surpass the length of half of the telophase spindle axis show mitotic, but not meiotic disturbances caused by non-separation of the elongated arms since the spindle axis in meiocytes is significantly longer than in mitotic cells. Plants carrying the elongated chromosome in homozygous condition are apparently slower growing and revealed reduced fertility, probably due to mitotic disturbances during early

embryogenesis. In *Vicia*, the adverse effects on mitosis and plant development increase with extension of the arm length above half of the spindle axis. In barley, the long arm of chromosome $6^{1/7}$ only slightly (0.47% ~0.3 μm) surpasses this limit. Further elongation of chromosome arm $6^{1/7}$L by recombination with a suitable translocation chromosome in future might yield even more severe effects as to mitotic non-separation of its sister chromatids, formation of micronuclei and disturbance of growth, development and fertility of carrier individuals.

iii. These results show that chromosome arms slightly longer than half of the average spindle axis length interfere with mitotic nuclear division and may cause cell death via chromatin deletion (Schubert *et al.* 1998a). Because dead cells arising from mis-division of meristematic cells may disturb tissue differentiation and thus affect the normal ontogenetic development of the organism concerned, half of the average spindle axis extension defines the upper tolerance limit for chromosome arm length. This is apparently a common rule for higher plants.

iv. While too small chromosomes preferentially may be incompletely transmitted through meiosis, chromosomes with long arms preferentially interfere with mitotic nuclear divisions since mitotic spindles are shorter than meiotic ones.

4 Summary

I. DNA composition and organization of barley centromeres

The BAC clone 03J24 (named BAC 7) was selected from a genomic barley BAC library to study sequence composition and arrangement of barley centromeres since it yielded positive FISH signals exclusively at the centromeric regions of all barley chromosomes and a hybridization pattern similar to that of genomic DNA after digestion with *Dra* I and Southern hybridization with pGP7 (a barley homologue of the centromere-specific *Sau3A9* element of sorghum) and BCS2 (barley variant of the *cereal centromere sequence*1) **(done by G. Presting)**.

The insert of BAC 7 (~23 kb), was found to harbour three copies of the *Ty3*/*gypsy*-like retroelement '*cereba*' flanked by LTRs of ~1 kb and a sequence with the predominant motif AGGGAG. While the *cereba* element shows high similarity to *gypsy*-like elements within centromeres of other cereals, the G+C-rich satellite is barley-specific. Both sequences constitute the major DNA components of all barley centromeres. The CCS1 sequences (Aragón-Alcaide *et al.* 1996) proved to be parts of LTRs, as proposed by Presting *et al.* (1998).

About 200 *cereba* elements of ~7 kb each, are present per barley centromere (Presting *et al.* 1998), indicating a considerably higher density than calculated for wheat or sorghum centromeres. The completeness of the *cereba* elements is a novelty when compared to that within centromeric clones of other cereals (see Langdon *et al.* 2000).

The functional meaning of *gypsy*-like retroelements within cereal centromeres is not yet clear. Although their number may be reduced below the detectability by FISH within mitotically and meiotically stable barley telosomics (T. R. Endo, pers. communication), they are apparently involved in recruiting CENP-A like kinetochore proteins in maize

(Zhong *et al.* in press). These results leave open the question whether or not kinetochore assembly at cereal centromeres is epigenetically regulated.

II. The upper chromosome size limit in barley

The observation of incomplete mitotic separation of sister chromatids of the recombinantly elongated arm $6^{1/7}$, the occurrence of micronuclei within meristematic cells and the reduced vigour of the recombinant cytotypes of barley correspond with the data reported for *Vicia faba* (Schubert and Oud 1997).

In barley, as well as in *V. faba*, chromosome arms which only slightly surpassed the length of half of the telophase spindle axis showed mitotic, but not meiotic disturbance based on non-separation of elongated arms since the spindle axis in meiocytes is significantly longer than in mitotic cells. Plants carrying the elongated chromosome in homozygous condition are slower growing and revealed reduced fertility, probably due to mitotic disturbances during early embryogenesis. In *Vicia*, the adverse effects on mitosis and plant development increase with extension of arm length (above half of the spindle axis). In barley, the long arm of chromosome $6^{1/7}$ only slightly surpasses this limit. Further elongation of chromosome arm $6^{1/7}$L by recombination with a suitable translocation chromosome in future might yield even more severe effects as to mitotic non-separation of its sister chromatids, formation of micronuclei and disturbance of growth, development and fertility of carrier individuals.

The data obtained show that chromosome arms only slightly longer than half of the average spindle axis length may interfere with mitotic nuclear division and may cause cell death via chromatin deletion (Schubert *et al.* 1998a). Because dead cells arising from mis-division of meristematic cells may disturb tissue differentiation and thus affect the normal development of the organism concerned, half of the average spindle axis

extension defines the upper tolerance limit for chromosome arm length. This is apparently a rule, at least for higher plants.

5 Zusammenfassung

I. DNA-Komponenten und deren Sequenzorganisation in Gerstezentromeren

Das Insert eines genomischen BAC-Klones der Gerste (BAC 03J24, hier BAC 7) ergab nach in situ Hybridisierung Signale ausschließlich an allen Gerstezentromeren. Nach Verdauung des Inserts mit *Dra* I und Southern-Hybridisierung mit dem Gerste-Homolog (pGP7) der zentromerspezifischen Sequenz *Sau3A9* aus Hirse bzw. mit dem Gerstehomolog (BSC2) der Getreidezentromer-spezifischen Sequenz CCS1 wurden identische DNA-Fragmente markiert wie nach entsprechender Hybridisierung von genomischer Gerste-DNA **(done by G. Presting)**.

Dieser BAC-Klon wurde für die weiteren Untersuchungen ausgewählt. Sequenzierung und DNA-Fingerprinting zeigten, dass das Insert von ~23 kb drei nahezu identische Kopien des *Ty3/gypsy*-ähnlichen Retroelementes '*cereba*' (centromeric retroelement of barley) sowie eine Sequenz mit dem vorherrschenden Motiv AGGGAG enthält. Während *cereba* hohe Ähnlichkeit mit *gypsy*-Typ-Elementen anderer Getreidezentromeren aufwies, erwies sich die GC-reiche Satellitensequenz als Gerste-spezifisch. Es wurde gezeigt, dass beide Sequenzen die Hauptkomponenten der zentromerischen DNA von Gerste bilden. Die CCS1-Sequenzen repräsentieren, wie von Presting *et al*. (1998) vermutet, die LTR-Sequenzen des Retroelements.

Ein Gerstezentromer enthält ca. 200 *cereba*-Elemente (Presting *et al*. 1998) und damit eine höhere Elementdichte als für die Zentromeren von Weizen und Hirse anhand der Sequenzdaten entsprechender Klone geschätzt wurde. Die Vollständigkeit der *cereba*-Elemente ist deutlich höher als die der entsprechenden Retroelemente in zentromerischen Klonen anderer Getreide (Langdon *et al*. 2000).

Die funktionelle Bedeutung beider Sequenzkomponenten für die Gerste-Zentromeren bleibt jedoch unklar. Obwohl beide Sequenzen in mitotisch und meiotischstabilen Telosomen der Gerste mengenmäßig unter die Nachweisgrenze für die in situ-Hybridisierung fallen können (T.R. Endo, pers. Mitteilung), sind zentromerspezifische Retroelemente und Tandem-Repeats des Mais anscheinend an der Bindung des zentromerspezifischen Kinetochore-Proteins CENP-A (Variante des Histons H3 in zentromerischen Nukleosomen) beteiligt (Zhong *et al*. in press). Damit bleibt die Frage, ob die Kinetochorposition bei Getreide epigenetisch reguliert ist, weiterhin offen.

II. Zur oberen Toleranzgrenze für die Chromosomengröße bei Gerste

Die Beobachtung einer unvollständigen mitotischen Schwesterchromatidentrennung für den rekombinant verlängerten Chromosomenarm $6^{1/7}$L der Gerste, das Vorkommen von Kleinkernen in meristematischen Zellen und die reduzierte Wüchsigkeit von Gerstepflanzen, die das verlängerte Chromosom enthalten, entsprechen den Befunden, die früher für die Ackerbohne erhoben wurden (Schubert and Oud 1997).

Bei Gerste wie bei Ackerbohne bewirken Chromosomenarme, deren Länge die Hälfte der durchschnittlichen Spindelachsenlänge während der Telophase nur geringfügig überschreitet, mitotische Störungen. Während der Meiose werden die Schwesterchromatiden der verlängerten Chromosomenarme auf Grund der längeren Spindelachse in Meiozyten problemlos getrennt. Pflanzen mit homozygot verlängerten Chromosomenarmen zeigen außer schwächerer Wüchsigkeit auch reduzierte Fertilität, die offenbar auf mitotische Störungen in frühen Embryonalstadien zurückgeht. Bei der Ackerbohne nehmen die nachteiligen Effekte auf die Mitose und die

Pflanzenentwicklung proportional mit der Länge der Chromosomenarme nach dem Überschreiten der Hälfte der Spindelachsenlänge zu. Der Chromosomenarm $6^{1/7}$L der Gerste überschreitet dieses Maß nur geringfügig. Eine weitere Verlängerung durch Rekombination mit einem geeigneten Translokationschromosom läßt eine Verstärkung der Effekte hinsichtlich der unvollständigen mitotischen Trennung der Schwesterchromatiden, der Kleinkernbildung und der Störungen der Pflanzenentwicklung und der Fertilität in den Trägerorganismen erwarten. Diese Ergebnisse zeigen, dass die beschriebene Chromosomenarmverlängerung auf Grund der Mitosestörungen über Kleinkernbildung (Chromatin-Deletion) zum Zelltod führen kann (Schubert *et al*. 1998a).

Da das Absterben meristematischer Zellen die Gewebedifferenzierung und die gesamtorganismische Entwicklung beeinträchtigt, stellt die halbe Spindelachsenlänge (während der Telophase) die obere Toleranzgrenze für die Chromosomenarmlänge dar. Diese Regel scheint, wie durch die Daten an Gerste bestätigt wurde, zumindest für höhere Pflanzen allgemein gültig zu sein.

6 Literature

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PUBLICATIONS IN CONNECTION WITH THE SUBMITTED DISSERTATION

HUDAKOVA S., MICHALEK W., PRESTING G. G., TEN HOOPEN R., DOS SANTOS K., JASENCAKOVA Z. AND SCHUBERT I. (2001) Sequence organization of barley centromeres. *Nucleic Acids Research* **29**: 5029-5035.

HUDAKOVA S., KÜNZEL G., ENDO T. AND SCHUBERT I. (2002) Barley chromosome arms longer than half of the spindle axis interfere with nuclear divisions. (submitted)

DECLARATION ABOUT THE PERSONAL CONTRIBUTION TO THE MANUSCRIPTS, FORMING THE BASIS OF THE DISSERTATION

The part **‚DNA composition and sequence organization of barley centromeres‘** is based on publication Hudakova et al. (2001) Nucleic Acids Research 29: 5029-5035. The work was done at Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben supervised by Prof. Dr. Ingo Schubert. The screening of a genomic BAC library of barley and most of the subcloning and shotgun sequencing of BAC 7 clone was done by Dr. Gernot Presting and Dr. Wolfgang Michalek. Experiments for fingerprinting by Southern blot analysis were started by Karla dos Santos and Dr. Rogier ten Hoopen. This work was later on continued and finished by myself. I did the remaining experimental work, sequence alignment and database comparison with centromeric sequences of other cereals and wrote the draft of the manuscript.

The part **‚The upper chromosome size limit in barley‘** is based on manuscript Hudakova et al. (submitted). The work was done at Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben in the frame of a project supported by the Deutsche Forschungs Gemeinschaft under supervision of Dr. Gottfried Künzel and Prof. Dr. Ingo Schubert. Original barley crosses were done by Dr. Gottfried Künzel. After initial support in performing banding technique by Prof. Takashi R. Endo, all experiments were done by myself including writing of the draft of the manuscript.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg noch einer andere wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre ferner, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertig habe.

Gatersleben, den .....

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