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# Molecular-cytogenetic characterization of a higher plant centromere/kinetochore complex

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**Abstract** The centromeric region of a telocentric field bean chromosome that resulted from centric fission of the metacentric satellite chromosome was microdissected. The DNA of this region was amplified and biotinylated by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR)/linker-adapter PCR. After fluorescence in situ hybridization (FISH) the entire chromosome complement of *Vicia faba* was labelled by these probes except for the nucleolus organizing region (NOR) and the interstitial heterochromatin, the chromosomes of *V. sativa* and *V. narbonensis* were only slightly labelled by the same probes. Dense uniform labelling was also observed when a probe amplified from a clearly delimited microdissected centromeric region of a mutant of *Tradescantia paludosa* was hybridized to *T. paludosa* chromosomes. Even after six cycles of subtractive hybridization between DNA fragments amplified from centromeric and acentric regions no sequences specifically located at the field bean centromeres were found among the remaining DNA. A mouse antiserum was produced which detected nuclear proteins of 33 kDa and 68 kDa; these were predominantly located at *V. faba* kinetochores during mitotic metaphase. DNA amplified from the chromatin fraction adsorbed by this serum out of the sonicated total mitotic chromatin also did not cause specific labelling of primary constrictions. From these results we conclude: (1) either centromere-specific DNA sequences are not very conserved among higher plants and are – at least in species with large genomes – intermingled with complex dispersed repetitive sequences that prevent the purification of the former, or (2) (some of) the dispersed repeats themselves specify the primary constrictions by stereophysical parameters rather than by their base sequence.

**Key words** Microdissection of plant centromeres · Subtractive hybridization · Fluorescence in situ hybridization · Indirect immunofluorescence · Immunoadsorption

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## Introduction

The centromere, usually the primary constriction, is the chromosomal domain of monocentric eukaryotic chromosomes and is responsible for their correct segregation during mitotic and meiotic nuclear divisions. Centromeres form the final points of adhesion for sister chromatids at the metaphase:anaphase transition, the attachment points for mitotic spindle fibers and the location for motor proteins mediating active chromosome movement during mitosis (for review see Earnshaw and Mackay 1994; Pluta et al. 1995; Sunkel and Coelho 1995). The kinetochore, previously a term synonymous to the centromere, now refers to a button-like proteinaceous structure resting on the surface of the centromeric chromatin, into which most of the microtubuli of the mitotic spindle attaching to the centromeric region are inserted (for review see Earnshaw 1991).

At present, detailed knowledge about DNA components essential for the functioning of centromeres is available only for yeasts. Centromeres of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* consist of three to four conserved centromeric DNA elements (CDEs) of about 250 bp all together, while in the fission yeast *Schizosaccharomyces pombe* the essential centromeric DNA consists of 44–100 kb with a central core of 4–7 kb (Clarke et al. 1993). These components are species-specific and do not function when transferred into another species (Heus et al. 1993).

For several insects and vertebrates satellite sequences are known to be located at centromeric regions. Murphy and Karpen (1995) have found a 220-kb island of complex DNA to be essential for the functioning of the centromere of a *Drosophila* minichromosome. For complete mitotic and meiotic stability this core had to be flanked on either side by approximately 200 kb of satellite DNA (AATAT). Despite extensive studies, especially ones on human alphoid sequences (see Haaf et al. 1992; Tyler-Smith et al.

1993; Page et al. 1995), the contribution of these sequences to centromere function has not yet been defined. In plants only very few cases are known of tandem repeats being specifically located at or around the centromeres (see Discussion).

Some specific nuclear proteins, such as CENPs A to F (*cen*tromeric *p*roteins) and INCENPs (*in*ner *cen*tromeric *p*roteins), are permanently or transiently (during mitosis) located at the centromeres/kinetochores of man and other mammals. For a few of these, putative homologs have been reported in yeast (for review see Sunkel and Coelho 1995). The first CENPs were detected by autoimmune sera from patients with CREST (*c*alcinosis, *R*aynauds phenomenon, *e*sophageal dysmotility, *s*clerodactyly and *t*elangiectasia) syndrome (Moroi et al. 1980; Brenner et al. 1981; Earnshaw and Rothfield 1985). A cross-reaction of CREST sera with plant kinetochores may occur (Mole-Bajer et al. 1990; Palevitz 1990; Houben et al. 1995). The centromere-associated proteins that were detectable by individual sera revealed species-specific differences among the electrophoretic mobilities of mammalian (Hadladczky et al. 1986) as well as plant species (Mole-Bajer et al. 1990; Houben et al. 1995). These proteins as well as the centromere-associated DNA sequences seem to be much more variable than the protein composition of the microtubuli (for review see Shaw 1994).

More detailed knowledge about the DNA and protein composition of plant centromeres is not only an indispensable prerequisite for understanding the mechanisms that regulate chromosome segregation during nuclear divisions and of the evolutionary convergence/divergence of this chromosomal 'organelle' between phyla but also for future construction of artificial plant chromosomes. We report here on our efforts to isolate and amplify DNA of plant centromeres using alternative strategies: (1) microdissection of centromeric chromatin (*Vicia faba*, *Tradescantia paludosa*); (2) immunoadsorption of centromeric chromatin from sheared total mitotic chromatin by kinetochore-specific antibodies (*V. faba*), each followed by PCR with degenerated and/or linker-adapter primers. DNA amplified from microdissected field bean centromeres was subtractively hybridized with DNA of an acentric region. The remaining fragments were cloned. After each step of the procedure the DNA was tested in situ for its centromere-specific location on homologous chromosomes. Additionally, the amplified DNA of the field bean centromeric region was hybridized in situ to chromosomes of *V. sativa* and *V. narbonensis*, and the specificity of the used antibodies used was checked by indirect immunofluorescence staining in situ and Western blot analysis of field bean nuclear proteins prior to immunoadsorption experiments.

## Materials and methods

### Preparation of chromosomes for microdissection, indirect immunofluorescence and in situ hybridization

Chromosome suspensions were prepared from synchronized root meristems of field bean (*Vicia faba*) lines with reconstructed karyotypes (ACB and 'Peru 14', Schubert and Rieger 1991) according to Schubert et al. (1993). The chromosome suspension was dropped onto slides for in situ hybridization and indirect immunofluorescence, or for microdissection on sterile coverslips and frozen on dry-ice. Air-dried preparations were used immediately or stored in glycerol at 4°C for up to several weeks for microdissection or in situ hybridization. For indirect immunofluorescence air-drying of the chromosomes had to be avoided.

For microdissection of the centromeric region, young buds of *T. paludosa* mutant 3-1-56 were squashed immediately in 45% acetic acid between two coverslips. This mutant is characterized by clearly delimited thin centromeric threads during pollen mitosis (Östergren and Östergren, unpublished) and was kindly provided by Dr. Lena Clapham, Uppsala.

For in situ hybridization, colchicine-treated (0.05%, 2 h) and fixed root-tip meristems of *T. paludosa*, *Vicia sativa* and *V. narbonensis* were softened in 1% cellulase (Onozuka R10) and 1% pectinase for 30 minutes at 37°C and squashed in 45% acetic acid on glass slides.

### Microdissection and preparation of the DNA of specific chromosome segments

An inverted microscope equipped with a micromanipulator was used for chromosome dissection (Houben et al. 1996). The centromeric region of the telocentric satellite chromosome of the field bean karyotype 'Peru 14', characterized by a stable centric fission, was dissected from 20 chromosomes (Fig. 1). These dissected regions and the corresponding acentric fragments were each collected in a 1-µl drop containing proteinase K (Boehringer, 0.5 mg/ml) in 10 m*M* TRIS-HCl (pH 8.0), 10 m*M* NaCl and 0.1% SDS overlaid with water-saturated paraffin oil. The DNA was amplified by DOP-PCR (degenerate oligonucleotide-primed polymerase chain reaction) according to Pich et al. (1994) using a degenerated primer ('MW-6'; Telenius et al. 1992). The delimited centromeric region of the gametophytic metaphase chomosomes of *Tradescantia* was treated likewise.

Alternatively, DNA of the microisolated centromeric region was manipulated according to a modified microcloning procedure of Johnson (1990). Chromosomal fragments were collected in a 1-nl collection drop of the same composition as above. The volume of the microdrops was estimated by the diameter of the drops. The droplets were extracted three times with water-saturated phenol and washed in a gentle stream of chloroform. The DNA was digested with the endonuclease *Sau*3A for 2 h at 37°C. After heat-inactivation of the enzyme the DNA fragments were ligated for 12 h at 13°C to a *Sau* 3A linker-adapter (24 mer/20 mer; Johnson 1990) under oil.

Finally, 1 µl of TE-buffer was added to the microdrop and transferred to 0.5 ml tubes for subsequent PCR amplification. The 20-mer component (5'GGATTTGCTGGTGCAGTACA3') of the linker-adapter was used as the primer. After denaturation for 5 min at 94°C, 2.5 U *Taq* DNA polymerase (Promega) was added; this was followed by 25 cycles of amplification (47°C 2 min, 72°C 3 min, 94°C 1 min) with a final primer extension step of 10 min. The extension time was prolonged by 3 s each cycle. For reamplification one-tenth of the first PCR products was amplified by a further 20 cycles. The final concentration of compounds in the PCR mix was 0.2 m*M* dNTPs, 6 µ*M* primer, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* TRIS HCl and 0,1% Triton X-100. An aliquot of the PCR products was checked by electrophoresis on 1.2% agarose gels.

### Subtractive DNA-DNA hybridization

The subtractive hybridization with magnetic beads was as described by Tagle et al. (1993) with some modifications. The DOP-PCR products of the centric and the corresponding acentric chromosome fragments were digested with *Sau*3A and purified using QUIAquick-spin columns (QUIAGEN). The digested DNA of the centric part was ligated to linker-adapter A (5'GGATTTGCTGGTGCAGTACA3', 3'CCTAAACGACCACGTCATGTCTAG5 ) and that of the acentric part to linker-adapter B (5'BioTTACCACGCTTCTATGTATC3', 3'AATGGTGCGAAGATACATAGCTAG5') (Johnson 1990). For hybridization, denaturated DNA fractions of the centric and the acen-

tric chromosome part were mixed in a ratio of 1:10 and incubated at 65°C for 16 h in 1.5 M NaCl, 50 m*M* Hepes (pH 7.5), 10 m*M* EDTA (pH 8.0) and 0.2% SDS under oil. The hybridization products were captured by streptavidin-coated magnetic beads M280 (Dynal). The beads (1 mg) were first washed three times in 6×SSC and resuspended in 1 *M* NaCl/1×TE-buffer. Then, hybridization mixture was added to the beads. After 1 min at room temperature the hybridized DNA bound to the beads was fixed by a magnetic particle concentrator while the unbound DNA of the centric chromosome region was pipetted off, precipitated and amplified by PCR using the 20-mer component of the linker-adapter A as primer. After denaturation for 10 min at 94°C, 2.5 U *Taq* DNA polymerase (Promega) was added; this was followed by 25 cycles of amplification (60°C 30 s, 72°C 90 s, 94°C 30 s) with a final primer extension step for 10 min. The autoextension time was 5 s. The final concentration of PCR compounds was 0.2 m*M* dNTPs, 0.3 µ*M* primer, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris HCl and 0.1% Triton X-100. Subtractive hybridization and amplification of the remnant DNA were repeated six times. From each subtraction cycle the PCR products were cloned into the *Bam*HI site of the vector $KS^+$ (Stratagene) and propagated in *E. coli* strain DH5alpha.

In total 1014 plasmids (169 from each subtraction cycle) were isolated for characterization of the recombinant clones, and the size of their inserts was determined. Undigested recombinant plasmid DNA (2 µg) was blotted onto Hybond $N^+$ membranes (Amersham). The filters were subsequently hybridized with labelled genomic *V. faba* DNA and with DNA of the acentric chromosome region and DNA of the centromeric chromosome region after each cycle of subtraction and amplification. The DNA probes were labelled by random priming in the presence of [$^{32}$P]-dCTP (Amersham).

### Isolation of genomic DNA and Southern blotting

Genomic DNA was isolated from leaf tissue and digested by the endonucleases *Sau*3A, *Hin*dIII, *Dra*I, *Bam*HI and *Eco*RI. After electrophoresis on 0.8% agarose gels the DNA was transferred by alkaline blotting onto nylon membranes.

### Fluorescence in situ hybridization (FISH)

The DNA isolated and amplified from the centromeric region was labelled with biotin 16-dUTP (Boehringer) by PCR according to Fuchs et al. (1994) using the 'MW-6' sequence or the 20 mer of the linker-adapter (see above) as primers. Fluorescent in situ hybridization was carried out as described by Fuchs and Schubert (1995). Hybridization of the biotinylated probes was detected by the streptavidin-fluorescein isothiocyanate(FITC)-antistreptavidin-system (Camon) using a fluorescence microscope with an appropriate filter system. Epifluorescence signals were recorded on Kodak Ektachrome film ASA400.

### Protein extraction and polyacrylamide gel electrophoresis

Chromosomes or nuclei isolated from 100 root tips were centrifuged and resuspended in 250 µl of 10 m*M* Tris/HCl (pH 8.0), 1 m*M* ethylene diaminoethanetetra-acetic acid disodium salt (EDTA), 12.5 µl 1 *M* dithiothreitole (DTT), 15 µl 100 m*M* phenylmethylsulfonyl fluoride (PMSF) and 62 µl 20% sodium dodecyl sulfate (SDS). After sonication on ice 1 µl Benzonase (Merck) was added. The solution was then incubated for 30 min at 37°C and finally diluted in gel loading buffer. The proteins were separated on 15% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane using a semi-dry blotter.

### Preparation of kinetochore-specific antibodies

A chromosomal protein fraction separated under denaturing conditions on 15% SDS-polyacrylamide gels was electro-transferred from gels to nitrocellulose membranes (Schleicher & Schüll BA85, 45 µm), and the kinetochore proteins on the membrane were recognized by indirect immunostaining using CREST serum S174 (Houben et al. 1995) in combination with alkaline phosphatase conjugated to anti-human immunoglobulin. The area containing kinetochore proteins of a molecular weight between 58 kDa and 65 kDa was excised from a part of the unstained Western blot membrane. The nitrocellulose pieces were grinded to fine powder under liquid nitrogen, resuspended with phosphate-buffered saline (PBS) and after supplementation with Freud's adjuvant used for immunization of the NMRI mice. Mice were primed and boosted four times over a period of 7 weeks by intraperitoneal injections of the same kinetochore proteins. Antibodies were raised from ascites fluid of immunized mice, and the immunoglobulin fraction was isolated by ammonium sulfate precipitation at 33% saturation followed by affinity chromatography on protein A-sepharose (Pharmacia).

### Indirect immunofluorescence

After three washes (10 min each) in PBS (pH 7.3), the slides were incubated for 1 h at room temperature in primary kinetochore-specific serum 5/1 diluted 1:50 in PBS in a humidified chamber. After an additional three washes in PBS the slides were incubated for 1 h at room temperature with FITC-conjugated anti-mouse IgG (Dianova) diluted 1:40 in PBS. Following three further washes in PBS, the preparations were counterstained with 4,6-diamino-2-phenylindole dihydrochlorid (DAPI, 1 µg/ml) and propidium iodide (1 µg/ml) in antifade.

### Western blot analysis

Primary antibody 5/1, diluted 1:300 in PBS for binding to separated and blotted centromeric proteins, was detected using secondary antibodies (anti-mouse IgG, MAB, 1:1000) conjugated to alkaline phosphatase and NPT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as a substrate. Blocking treatment with BSA (bovine serum albumin) and washing of membranes was performed following the manufacturer's instructions (Amersham manual).

### Isolation of centromeric DNA fragments via immunoadsorption

Chromosomes of 200 root-tip meristems were isolated as described above, centrifuged and resuspended in 200 µl of ice-cold 1×PBS (pH 7.3) containing 400 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* PMSF, 5 m*M* EDTA, 0.1% Triton X100, 0.1% Nonidet P-40 and 2% BSA. The suspension was sonicated five times for 30 s until the size of the chromosome fragments was less than 0.5 µm when checked under the microscope.

Tosylactivated magnetic Dynabeads M-280 (Dynal) were loaded with purified anti-kinetochore antibody 5/1 according to the manufacturer's instructions. Antibody-loaded magnetic beads (100 µl) and the sonicated chromosome suspension (200 µl) were incubated together for 2 h under gentle rolling. Unbound chromosome fragments were pipetted off, while the beads with bound fragments were kept at the wall of the Eppendorf tube by means of a magnetic particle concentrator. After five washes of 10 min each at room temperature, each in 200 µl 1×PBS with 0.1% Tween 20 and 2% BSA, the beads were washed in 1×PBS, 0.1% Tween 20 for 10 min at room temperature and treated with 0.2 mg proteinase K in 0.5 ml of 10 m*M* Tris-HCl (pH 8.0), 10 m*M* NaCl and 0.1% SDS for 8 h at 50°C. The DNA was then phenol/chloroform-extracted and ethanol-precipitated. The precipitate was resuspended in 5 µl TE, and the DNA amplified by DOP-PCR.

## Results

### Attempts to isolate centromere-specific DNA of plants via microdissection and subtractive hybridization

From a telocentric-satellited field bean chromosome (Fig. 1), which represents one of the products of a centric

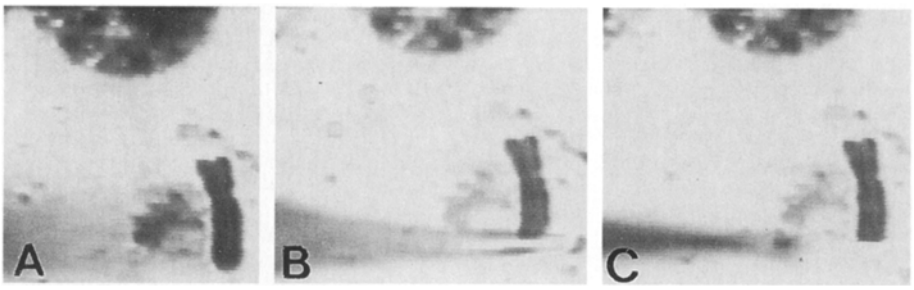


**Fig. 1** Microdissection of the centromeric region of the telocentric satellite chromosome of field bean karyotype 'Peru 14' before **A**, during **B** and after **C** isolation by means of a microneedle

fission of the metacentric satellite chromosome (Schubert and Rieger 1990; Schubert et al. 1995), the centric end (<1 μm) and the complementary acentric chromosome part were isolated by microdissection. The DNA of both isolated chromosome segments was amplified separately by DOP-PCR; the DNA of the centromeric region was additionally amplified by linker-adapter PCR. The amplified DNA of the microisolated chromosome regions formed a smear within the range of 0.1–1.8 kb on agarose gels.

After the in situ hybridization of labelled DNA of the centromeric region all of the chromosomes revealed disperse signals except for the nucleolus organizing region (NOR) and the interstitial heterochromatic regions. These latter areas were more or less free of hybridization signals (Fig. 2). The labelling pattern was identical when DNA was amplified via linker-adapter PCR or via DOP-PCR. Since the hybridization patterns on the Southern filters were similar for labelled DNA of both the centric and acentric chromosome parts (Fig. 3), it was concluded that both regions harbour the same disperse repetitive DNA sequence(s) that are responsible for the hybridization patterns observed. Three fragments (Fig. 3, I: A, C) which gave hybridization signals with the DNA of the centric region were isolated from the gels. After biotinylation and in situ hybridization they yielded disperse signals.

Assuming that functional centromeric sequences rather than dispersed repetitive sequences should be conserved between related species during evolution, we hybridized labelled DNA probes of the centromeric region of the field bean to chromosomes of *V. sativa* and *V. narbonensis*. This yielded a low number of dispersed signals over the entire chromosome complements without any distinct label at the primary constrictions (data not shown). The biotinylated DNA amplified by DOP-PCR from the microdissected *Tradescantia* centromere (Fig. 4) hybridized at a uniform density along all of the chromosomes of that species (Fig. 5). To enrich and purify centromere-specific sequences, we performed six cycles of subtractive hybridization between DNA probes amplified from centromeric and those from acentric chromosome regions.

The remaining DNA fractions supposed to be enriched in centromere-specific DNA were cloned after each cycle. In total, 1014 clones were used for subsequent dot-blot hybridization with labelled probes of total genomic DNA and DNA of both the centromeric and acentric regions after each cycle of subtractive hybridization. Single clones which revealed strong signals with total genomic DNA and

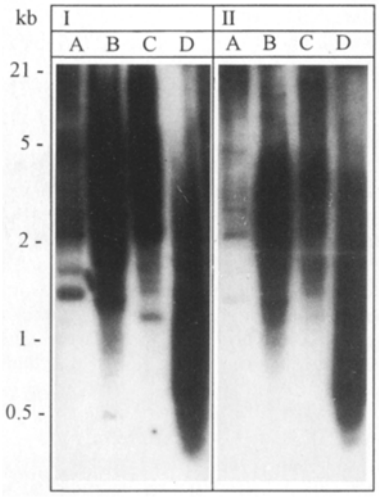


**Fig. 3** Sequential Southern hybridization of *A BamHI*-; *B HindIII*-; *C EcoRI*-; and *D Sau*-3A-digested genomic *V. faba* DNA with labelled DOP-PCR products of the centric chromosome region (*I*); and with labelled DOP-PCR products of the acentric chromosome region (*II*)

DNA of the centromeric region but no or only weak signals with DNA of the acentric region were selected for further in situ hybridizations. None of the 28 clones selected in this way yielded pronounced centromere-specific signals on *V. faba* chromosomes. In most cases disperse labelling was observed. Only 1 clone (A160) with an insert of 84 bp revealed a faint signal at the homologous centromeric region (on chromosome I of karyotype ABC, data not shown). This clone was used for screening a phage library of *V. faba* genomic DNA with larger inserts. However, homologous clones with an insert size of approximately 20 kb resulted in a disperse in situ hybridization pattern comparable to that of the centromeric DNA probe before subtractive hybridization.

## Attempts to isolate centromere-specific DNA via immunoadsorption by means of a kinetochore-specific antibody

Previously we reported a CREST serum (S 174) which specifically detects antigenic proteins of *V. faba* kinetochores (Houben et al. 1995). Since there was not a sufficient

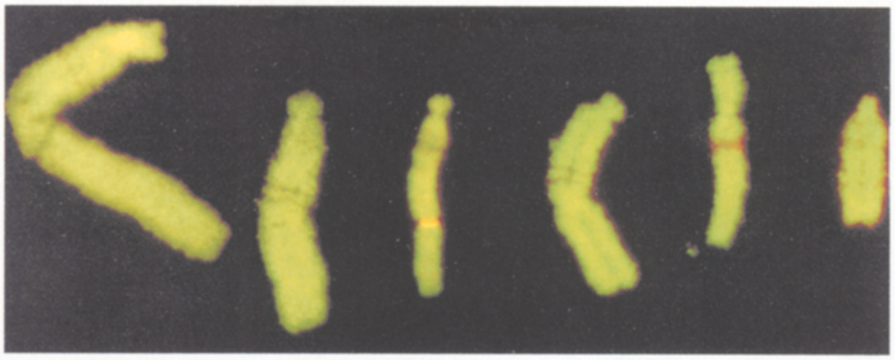

**Fig. 2** FISH of biotinylated PCR products obtained with linker-adapter primers from DNA of the centromeric region of the telocentric satellite chromosome of field bean karyotype 'Peru 14' to metaphase chromosomes I – VI of karyotype ACB

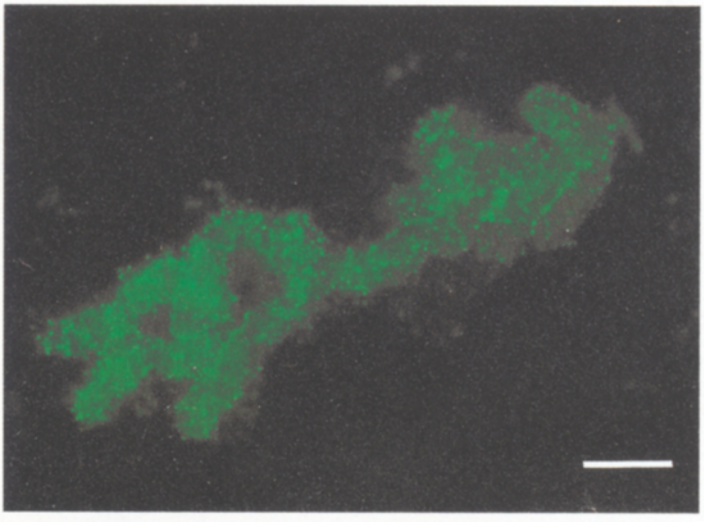

**Fig. 5** Metaphase chromosomes of *T. paludosa* after FISH with labelled DNA amplified from the microdissected centromeric region. *Bar*: 5 µm

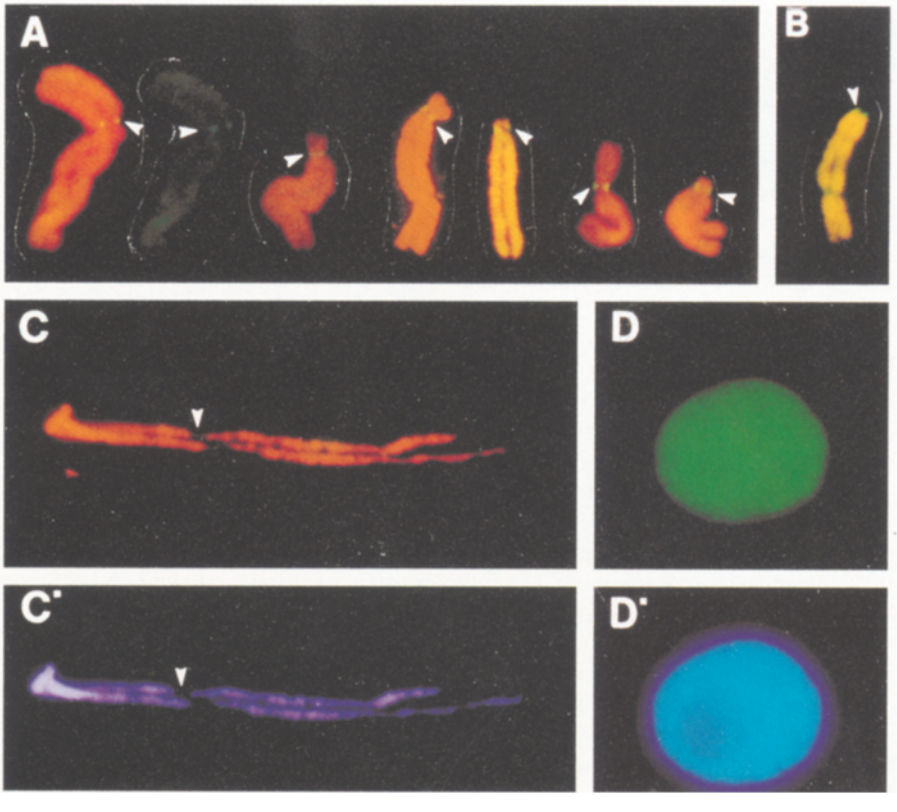


**Fig. 7A–D** Indirect immunofluorescence staining with the antiserum 5/1. **A** Examples of *V. faba* chromosomes I-VI of karyotype ACB showing one fluorescent spot at the centromeric region (*arrowheads*) of each chromatid; photographed using a filter combination specific for FITC plus propidium iodide. The second chromosome I (*second* from *left*, **A**) and the nucleus (**D**) were photographed using the fluorescein-specific filter only. **B** A telocentric satellite chromosome of the karyotype 'Peru 14'. **C** Stretched *V. faba* chromosome showing fluorescence along the entire centromere region. **D** Interphase nucleus showing weak disperse labelling. **C′** and **D′** are the same as in C and D after DAPI staining

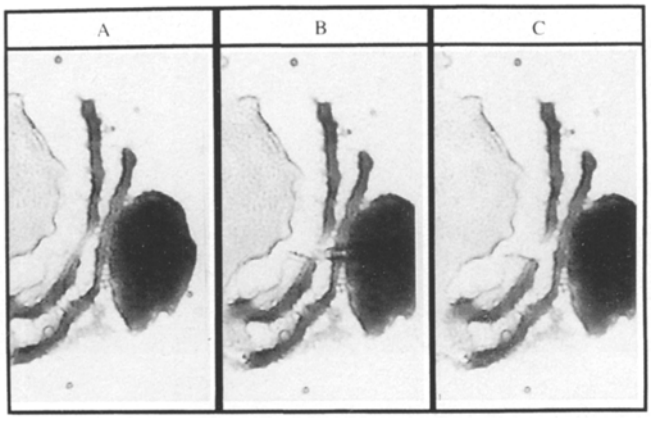


**Fig. 4A–C** Sequence of microdissection of the delimited centromeric region from a chromosome of *T. paludosa* during pollen grain mitosis (**A–C**)

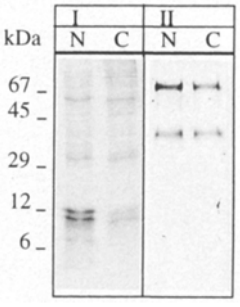


**Fig. 6** Protein profiles of interphase nuclei (*N*) and metaphase chromosomes (*C*) of *V. faba* separated electrophoretically and stained with Coomassie brillant blue (*I*). Western blot of the same gel after reaction with the antiserum 5/1; proteins of 33 kDa and 68 kDa are recognizable (*II*)

amount of this serum to perform immunoadsorption experiments in order to enrich kinetochore-specific chromatin, it was necessary to prepare a new antibody specific for *V. faba* kinetochores. With this purpose in mind, chromosomal proteins were isolated and separated by SDS-polyacrylamide gel electrophoresis. A comparison of proteins isolated from interphase nuclei and metaphase chromosomes revealed similar profiles (Fig. 6). Since the two major kinetochore-specific proteins of *V. faba* detected by CREST serum S 174 (Houben et al. 1995) have a molecular weight of 64 kDa and 68 kDa, respectively, proteins of corresponding size were isolated from protein-loaded membranes and used for immunization of the mice.

Sera from 25 immunized mice were screened for their ability to detect kinetochore-specific antigens on field bean chromosomes by indirect immunofluorescence. With 8 of these sera no clear signals were found on the chromosomes, while 16 of them resulted in dispersed labelling of the entire chromosome complement. One antiserum (5/1) clearly labelled the centromeric region with a low background staining of the remaining mitotic chromosomes. Fluorescent spots at the primary constriction of both chromatids were regularly visible. The interphase nuclei revealed a homogeneous fluorescence (which was reduced but not abolished in controls without serum 5/1), but no pronounced labelling of prekinetochores (Fig. 7). This is indicative of a protein that accumulates at kinetochores during mitosis and is comparable to CENP-E and CENP-F of human chromosomes (Yen et al. 1991; Liao et al. 1995) and to a protein of *Allium,* homologous to a mammalian centrosomal antigen (Schmit et al. 1994) and to a mitotic phosphoprotein of the field bean (Binarova et al. 1993).

Serum 5/1 was also used to identify antigenic kinetochore proteins by Western blot analysis. Of the separated proteins of the chromosomes and nuclei two proteins of a relative molecular weight of 33 kDa and 68 kDa were detected by the serum (Fig. 5). These proteins differ in size from human centromeric proteins as well as from those of *Haemanthus* detected using CREST serum EK (Mole-Bajer et al. 1990). The larger one corresponds in size to the 68-kDa protein that was detectable via Western blot analysis by the CREST serum S174 (Houben et al. 1995).

Similar to the strategy of Hadlaczky et al. (1991) and Ouspenski and Brinkley (1993), a purified antibody preparation of the kinetochore-specific antiserum (5/1) was used for attempts to enrich a centromere-specific fraction from sonicated chromatin by immunoadsorption. The DNA isolated from the adsorbed chromatin fraction was amplified by DOP-PCR, biotinylated and used for in situ hybridization to *V. faba* chromosomes. Comparable to the probes derived from the microdissected centromeric region, this DNA labelled all chromosomes uniformly except at the NOR and the interstitial heterochromatin.

## Discussion

It could be demonstrated that plant kinetochores also contain proteins that accumulate at mitotic centromeres in addition to those that remain in that position during interphase.

Our efforts have shown that plants with large genomes contain disperse repetitive sequences within their primary constrictions. However, it was not possible to isolate DNA sequences specifically located at the centromeres of these plants. Neither microdissection and subsequent subtractive hybridization between amplified DNA fragments of centromeric versus acentric chromosome regions, nor immunoadsorption of centromeric chromatin fragments by kinetochore-specific antibodies yielded purified DNA sequences that were located at field bean centromeres exclusively and thus could be considered as candidates for centromere-specific sequences.

What could have been the reasons?
– The centromere-specific sequence might selectively have been excluded from PCR amplification by the use of the degenerated MW-6 primer and by the distribution of recognition sites for the restrictase *Sau*3A used for DNA digestion prior to ligation of the linker-adapters. Though not very probable, this possibility cannot be excluded.
– Because it is usually impossible to delimit exclusively the centromere from the remaining chromatin, too many nonspecific sequences may have been dissected out in addition to the centromere. However, the results obtained

with DNA from microdissected centromeric regions of *Tradescantia paludosa* mutant 3-1-56 clearly demonstrate that disperse repetitive sequences themselves may constitute one component of the primary constrictions of higher plants.

– A single unique sequence, even if it covers several kilobases, could have escaped from detection since the cloned amplification products of a few hundred base pairs cannot yield resolvable hybridization signals in situ (Jiang and Gill 1994). The chance to isolate this sequence completely and free of flanking repetitive ones from a phage library is rather low. However, the assumption that a single unique sequence organizes a structure of the dimensions of a centromere also does not seem to be realistic.

– The centromere specificity could be mediated by one or more short repeats embedded and interspersed by disperse repetitive sequences. Due to their high complexity within large genomes, the latter cannot be removed completely, neither by conventional subtractive procedures nor by prehybridization with total genomic DNA in excess (Fuchs et al. 1996). A single such sequence remaining in the probe will yield disperse hybridization signals. The same happens when disperse repetitive sequences that usually flank coding sequences in the field bean genome are not removed from phage clones before FISH (Fuchs et al. 1994).

– The failure to purify centromere-specific sequences by immunoadsorption could have been due either to disperse repetitive sequences within the centromeric chromatin fraction or to the weak cross-reaction of serum 5/1 with noncentromeric chromatin sufficient to contaminate the probe by (a) dispersed repeat(s). Similar observations were made by Ouspenski and Brinkley (1993).

– Finally, it cannot be excluded that a centromere-specific DNA, based on a distinct base sequence, does not exist at all in plants. Vig (1994) has speculated that the physical appearance of the centromere is based on stereophysical properties, particularly the curvature of DNA, recognizable by a proteinaceous binding factor ('centromerase'), rather than on a specific base sequence in this region. It is also possible that a special folding structure of the nucleosomal DNA protein complex mediated by posttranslational histone modifications (Loidl 1988; Turner 1993) is essential for functional centromeres but does not strictly depend on a defined DNA sequence.

The hybridization patterns of the DNA probe from the field bean centromeric region to chromosomes of the related species *V. sativa* and *V. narbonensis* support the latter assumption. The lack of distinct signals at the primary constrictions of these species indicates the absence of a centromere-specific and evolutionary conserved sequence that remained cryptic in *V. faba* due to the uniform labelling by species-specific dispersed repeats present within the probe.

The chromosomes of many animal species are characterized by pronounced centromeric/pericentromeric heterochromatin (van Holde 1988). The major DNA component of this heterochromatin is usually formed by – not very conserved (Archidiacono et al. 1995) – tandemly repeated satellite or other simple sequences. The presence of these sequences is supposed to be a necessary (Haaf et al. 1992) but not sufficient (Page et al. 1995; Murphy and Karpen 1995; Sullivan and Schwartz 1995) condition for a functional centromere. However, the association of the minor satellite with the centromere of mouse chromosomes was recently questioned by the data of Vig et al. (1994). In plant chromosomes centromeric heterochromatin is usually not that pronounced. In *V. faba* primary constrictions revealed small Giemsa bands only after extended barium hydroxide pretreatment, when the structure of euchromatin and interstitial heterochromatin already appeared to be disrupted (Döbel et al. 1978). Also, after heat denaturation the structural integrity of centromeric regions remains well-preserved (Houben, unpublished). Tandemly repeated sequences have been found to be located at positions of interstitial heterochromatin but not at centromeres in *V. faba* (Fuchs et al. 1994). Although there are a few reports of pericentromerically located repetitive sequences in plants with small genomes (Maluszynska and Heslop-Harrison 1991; Murata et al. 1994; Harrison and Heslop-Harrison 1995; Alfenito and Birchler 1993; Leach et al. 1995) it remains to be elucidated whether or not they are of importance for the centromere function.

In our opinion the most reasonable alternative interpretations of available data are: (1) either centromere-specific DNA sequences are not very conserved in higher plants and may occur intermingled with complex dispersed repeats; or (2) some dispersed repeats themselves specify the centromeres by stereophysical parameters (and specific interaction with kinetochore proteins) rather than simply by base sequence.

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