Plant Molecular Biology Reporter
Volume 9(1) 1991

pages 21-30

Protocol

Isolation of a Sequence Common to A- and B-Chromosomes of Rye (*Secale cereale*) by Microcloning

Michael J. Sandery, John W. Forster, Simon R. Macadam, Richard Blunden, R. Neil Jones, and Stephen D. M. Brown*

(MJS) Centre for Plant Biotechnology, Bond University, Gold Coast, Queensland 4229, Australia. (JWF, SRM, RB, RNJ) Genetics and Crop Biotechnology Group, Department of Agricultural Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DD, Wales, UK. (SDMB) Department of Biochemistry and Molecular Genetics, St. Mary's Hospital Medical School, Paddington, London W2 1PG, UK.

Key Words: B-chromosome, microcloning, rye, evolution.

Abstract: The techniques of microdissection and microcloning have been applied to the isolation of B-chromosome DNA from rye. We have identified a DNA sequence on the rye B-chromosome which is homologous to an A-chromosome sequence, and which is dispersed and moderately repeated on the A- and B-chromosomes. This demonstrates that the rye B-chromosome is heterogeneous in the nature of its DNA sequence composition, containing sequences which are present on the A-chromosomes in addition to those not present on the A-chromosomes.

Supernumerary B-chromosomes are a common cause of numerical chromosome polymorphism in many plant and animal species. In rye, the B's are additional to the basic complement of 2n=14, which are termed the A-chromosomes. The B's are dispensable for growth, fail to pair with the A's at meiosis, and show irregular modes of

Abbreviations: UV, ultraviolet light; PEG, polyethylene glycol; DEAE, diethylaminoethyl; PCR, polymerase chain reaction.

*To whom correspondence should be addressed.

inheritance due to directed non-disjunction towards the generative nucleus and the functional egg nucleus. Plants with high numbers of B's show deleterious effects on characters such as seed gemination and the size of mature plants. Although the effects and behavior of B-chromosomes have been well studied in many species (for review see Jones and Rees, 1982), little is known about the nature of the DNA in B-chromosomes. In an effort to shed some light on the nature and origin of these chromosomes, we have attempted to isolate and clone DNA from B-chromosomes of rye (*Secale cereale*) using several different methods.

We have previously identified a sequence which is highly repeated on the rye B-chromosome and which has not been detected on the A-chromosomes (Sandery et al., 1989), suggesting that a proportion of rye B-chromosome DNA is markedly different to A-chromosome DNA. This sequence was identified by comparison of restriction enzyme patterns of DNA from OB and +B rye plants. To date, this is the only direct evidence we have of the nature of the DNA of B-chromosomes in rye.

Direct analysis of B-chromosome DNA is best performed using a highly enriched or, preferably, pure source of B-chromosomes. To achieve this, we have used microdissected rye B-chromosomes, isolated from chromosome spreads prepared from naturally synchronous rye meiocytes. Microdissection, in conjunction with microcloning, was first described for *Drosophila melanogaster* polytene chromosomes (Scalenghe et al., 1981). Microcloning may be defined as the *in-vitro* manipulation of DNA sequences in nanoliter volumes using appropriate amounts of vector and enzymes. The technique involves the physical removal of the desired chromosome from a fixed preparation of chromosomes, using specially constructed microinstruments. DNA is extracted from the pooled chromosomes and cloned into an appropriate vector. The technique has been refined and extended to metaphase chromosomes of mouse (Fisher et al., 1985) and man (Bates et al., 1986).

The size and behavior of rye B-chromosomes make them ideal candidates for microdissection, since they are easily identified even in unstained material. Here we report on the use of microdissection and microcloning in isolating and cloning rye B-chromosome DNA, and we show that the rye B-chromosome, in addition to carrying sequences not detectable on the A-chromosome, also consists of sequences present on the A-chromosomes.

Materials and Methods

Material for microdissection

Plants carrying B-chromosomes were from a population of Japanese rye established by Kishikawa (1965). Anthers were excised from developing spikes and examined for the presence of cells in meiosis. When such an anther was found, remaining anthers from that floret were fixed in 3:1 ethanol:acetic acid for 3 to 5 minutes, and dissected in a drop of 20% (v/v) acetic acid on a coverslip and squashed. The coverslips were snap-frozen in dry ice, the top coverslip was removed with a scalpel and the coverslip carrying meiotic chromosomes was stored in absolute ethanol at -70°C.

Equipment for microdissection

The microforge for making instruments and the micromanipulating equipment were supplied by de Fonbrunne. The microdissections were performed under oil using a 32X phase contrast objective on a Leitz phase contrast microscope, with a long focal length to accommodate the oil chamber. Microneedles, micropipettes and coverslips used in the microcloning steps were siliconized prior to use.

Solutions for microcloning

Extraction buffer: 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.5% SDS, 0.5 mg/mL proteinase K

10x *Eco* RI Buffer: 1.0 M NaCl, 100 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM 2–ME

Phenol: equilibrated with 0.1M Tris buffer (pH 8.0), then with 1x *Eco* RI buffer.

Paraffin oil: equilibrated with 1x *Eco* RI buffer.

Microdissection and microcloning procedure

B-chromosomes were identified from meiotic cells, and were dissected and cloned by the method of Fisher et al. (1985). This involves the following steps:

- Three cover slips were set up adjacent to one another on the oil chamber. The first coverslip (A) carried the chromosomes to be dissected, while the third (C) carried a 1.0-µL drop of extraction buffer. This coverslip was also inverted on the oil chamber, which was filled with paraffin oil and placed on the microscope slide.

- A clean micropipette was used to dispense two 1.0-nL drops of extraction buffer onto the second coverslip (B). In all subsequent manipulations coverslip C was used as a supply slip to the manipulations on coverslip B. A microneedle was placed in the micromanipulator. The tip of the needle was positioned just underneath and slightly to one side of the B-chromosome. Using the microscope stage control, the oil chamber was moved so that the B-chromosome passed over the tip of the needle. At the same time the needle was moved so as to contact the chromosome, then moved down and away, dragging the chromosome with it.
- The chromosome was transferred from the tip of the needle into one of the collection drops on coverslip B. This process was repeated until sufficient B's had been collected. The two drops were then fused by moving the needle across them.
- Incubations of the microdissected material were performed by placing the entire oil chamber (with coverslips) at the desired temperature. The oil chamber was changed after each phenol extraction.
- In the microcloning, the material was first treated with extraction buffer for two hours at 37°C.
- The droplet was then extracted with phenol three times by dispensing the phenol so as to engulf the microdrop. The phenol was then removed and discarded.
- Digestions with *Eco* RI were performed at 37°C for two to three hours. A 2.0-nL drop of 50 U/μL *Eco* RI in 2x *Eco* RI buffer was fused with the 2.0-nL reaction microdrop.
- Three further phenol extractions were performed.
- For ligation, a 4.0-nL drop of 160 pg/nL λgt10 arms and 4.0 mM ATP was fused with the reaction drop. An 8.0-nL drop of 1.0 U/μL T4 ligase was added. The final droplet had a volume of 16 nL, just visible to the naked eye. Ligation was performed at 4°C for 12-18 hours.
- The droplet was removed into a microtube and packaged *in vitro* using the Gigapack Plus system (Stratagene, Inc.).
- Recombinant phage were plated on the selective host C600 *hflA* (Young and Davis, 1983) and identified as clear plaques.

DNA isolation and gel electrophoresis

Plants were screened for the presence of B-chromosomes by root tip analysis, and DNA was isolated from leaf material according to the method of Raeder and Broda (1985). Restriction digests were performed with a 5- to 10-fold excess of enzyme, according to the supplier's

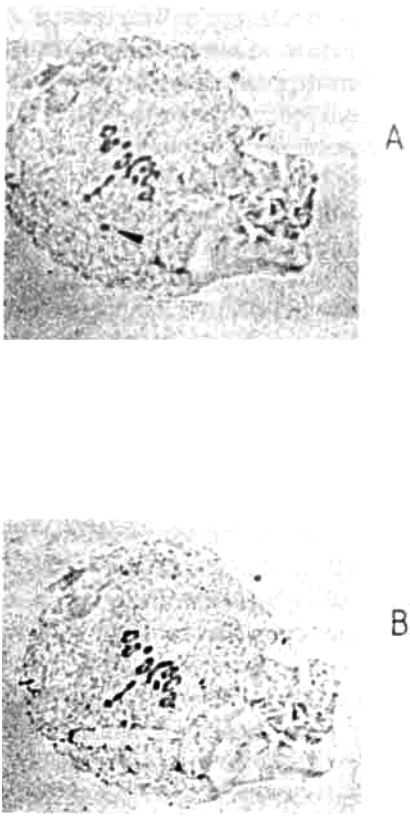

Fig. 1. Microdissection of a rye B-chromosome from a meiotic metaphase I spread. (A) Prior to microdissection and (B) after microdissection. The position of the B-chromosome is indicated by the arrow.

recommendations. Digested DNA was run on 0.7% agarose gels at 5 V/cm, stained with ethidium bromide and photographed under UV illumination. DNA was transferred to Zetaprobe nylon membranes by the method of Reed (1986a) and was prehybridized, hybridized and washed according to Reed (1986b) with PEG6000 instead of dextran sulphate. Autoradiography was at -70°C with intensifying screens.

Probe preparation

Phage DNA from microclone λM2.1 was purified by lysis of liquid culture (Davis et al., 1986), and the 2-kb *Eco* RI insert in this clone was isolated by DEAE membrane elution. Insert DNA was oligolabelled with[^{32}P]-dGTP (Feinberg and Vogelstein, 1984) and purified by precipitation with ammonium acetate.

Results

In order to microdissect chromosomes, it is essential to have chromosome spreads in which the overlapping of chromosomes is minimal, and in which the chromosome to be dissected is both recognizable and accessible. Chromosomes prepared from pollen mother cells are much more accessible than those prepared from root tips, since the chromosomes are often seen spread over the slide instead of remaining within individual cells. In addition, the meiocytes are highly synchronized at metaphase I and anaphase.

Meiocytes containing a single B-chromosome were selected for microdissection. One of the diagnostic features of B-chromosomes is their failure to pair with any of the A-chromosome complement (Jones and Rees, 1982). A single B-chromosome remains unpaired as a univalent in metaphase I cells, and can often be seen lagging behind the separating sister chromatids of the A-chromosomes at anaphase I. Thus the B-chromosome can be most readily distinguished in IB genotypes.

Figure 1 shows the metaphase I chromosomes from a pollen mother cell from a +B genotype of rye. In this case, the chromosomes have remained within their cell, and show good morphology. We have used fixation times of between three to five minutes, having found that this was the shortest time which yielded fixed chromosomes of suitable morphology. Due to the rapid growth of the rye plants, chromosome preparations had to be stored for between four to nine weeks before microdissection, with no apparent effect on morphology.

It is noticeable when performing dissections on material such as this that, in addition to removing the chromosome, some 'background' of cellular material is also collected. This material is visible in Figure 1. This seems to be a feature of microdissections, even when performed on lymphocyte chromosomes and does not seem to interfere with the efficiency of the procedure.

A small number of clear plaques (ca. 20) were obtained by plating the microcloning packaging on the selective host C600 *hflA*. A control plating on C600 confirmed that clear-plaque clones constituted c. 20% of the phage population, which is well above the proportion expected due to mutation in the *imm434* repressor gene of λgtl0. A number of these microclones were analyzed by preparation of phage DNA. The majority failed to produce a visible insert after *Eco* RI digestion and electrophoresis on 1% agarose gels. We have also performed PCR with primers flanking the λgtl0 *Eco* RI cloning site on several of these clones, again failing to produce a visible insert. We assume that either these phage clones are mutations or, more likely, they contain inserts too small to be resolved by physical analysis. It may be necessary to perform DNA sequencing across the *Eco* RI cloning site in order to confirm this hypothesis.

One of the putative microclones, λM2.1, was found to contain a 2-kb insert. If this sequence was unique to the B-chromosome, it should show exclusive hybridization to the digests of DNA from the plant carrying four B-chromosomes and that containing an isochromosome for the long

arm of B (assuming that the sequence is located on the long arm of B). In addition, the intensity of hybridization observed should be correlated with the number of B-chromosomes. In fact, the clone shows apparently equal hybridization to the digests from both OB and +B genotypes. We have modelled the expected intensities of hybridization given the distribution of B-chromosome-located clones (J.W.F., M.J.S. and S.R. Barnes, unpublished) and the observed pattern is consistent, given equal loading of DNA between OB and +B genotypes, with the isolation of a repetitive sequence present with equal abundance on the A's and the B's. The λM2.1 insert shows an unusual hybridization pattern (Fig. 2). The majority of the hybridization is to the high molecular weight (relic) DNA, but there is a diffuse hybridization to most molecular weight positions, with some faint internal banding. This indicates that some of the sequence may be in a tandem arrangement on the chromosome, but also some is in a dispersed arrangement. A faint ladder pattern is observed with the enzyme *Xba* I, with bands spaced every 400 bp. Such a pattern could derive from the presence of a single *Xba* I site in the basic unit of the tandem cluster, with site loss polymorphism generating the ladder. At this level of resolution, the sequence seems to show similar organization in both the A- and B-chromosomes.

Discussion

We have demonstrated that the B-chromosome of rye contains DNA sequences which are found on the A-chromosomes, in addition to those sequences which seem to be specific to the B-chromosome. This may suggest that the composition and distribution of DNA sequences on B-chromosomes is the same as in A-chromosomes, a notion which has also been supported by earlier investigations (Rimpau and Flavell, 1975; Timmis et al., 1975). However, these same studies have also concluded that the existence of repeated sequences specific to the B-chromosome was unlikely, whereas we have identified a B-specific repeated sequence. These results highlight the need to isolate and investigate B-chromosome DNA free from A-chromosome contamination to gain an accurate picture of B-chromosome structure. The microdissection of B-chromosomes is a simple and effective method to obtain a pure source of chromosomes.

The most popular model for the origin of B-chromosomes proposes that the B-chromosomes of most species are derived from the A-chromo-

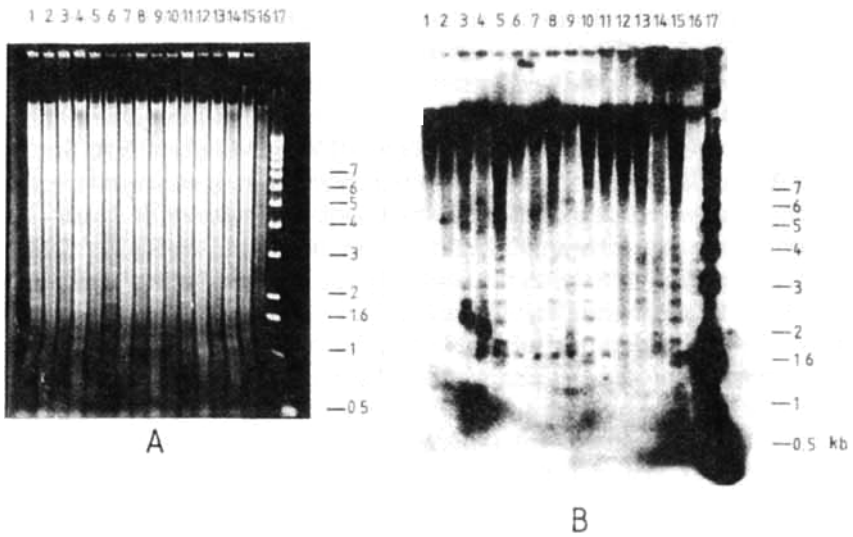

Figure 2. Results of the hybridization of the 2-kb *Eco* RI insert from microclone λ M2.1 to a panel of Japanese rye digests. (A) Electrophoretogram of rye DNA. Lane 1, OB + *Eco* RI; 2, OB + *Dra* I; 3, OB + *Bam* HI, 4, OB + *Hind* III; 5, OB + *Xba* I; 6, 4B + *Eco* RI; 7, 4B + *Dra* I; 8, 4B + *Bam* HI; 9, 4B + *Hind* III; 10, 4B + *Xba* I; 11, 1B-isoL + *Eco* RI; 12, 1B-isoL + *Dra* I; 13, 1B-isoL + *Bam* HI; 14, 1B-isoL + *Hind* III; 15, 1B-isoL + *Xba* I; 16, 4B + *Dra* I; 17, 1-kb DNA ladder. (B) The gel in (A) blotted onto a nylon membrane and probed with the λM2.1 insert.

somes (Dover, 1975; Dover, 1976b; Amos and Dover, 1981). This model is based on the similar DNA profiles of A's and B's. Our evidence provides some support for this model, although a more extensive analysis of B-chromosome sequences is needed.

The application of microdissection and microcloning has great potential for the analysis of plant genomes by providing large numbers of chromosome-specific and region-specific probes. At present, the microcloning procedures for plant chromosomes require modification to increase the yield of microclones. Although the amount of dissected DNA (20 to 30 pg per microcloning) was sufficient to yield many microclones, the crucial factors in determining both the yield and the size of microclones are thought to be the fixation time and the type of plant material used in preparing chromosomes. The very low number of

viable microclones generated in this experiment is probably related to processes described by Brown and Greenfield (1987), who proposed that in mammalian microcloning experiments a deleterious event occurring on average every 100 bp renders many sequences unclonable. It is speculated that this event may be due to a hydrolytic depurination of DNA that would occur during acid fixation of metaphase chromosomes and base hydrolysis of DNA when restored to neutral pH. The relatively long fixation times involved in these plant microcloning experiments may lead to numerous deleterious events of this sort, with the consequence of a very low yield of microclones. In addition, we cannot rule out the possibility, given the hybridization of the λM2.1 insert to DNA from both OB and +B DNA, that the clone is derived from underlying nuclear material fortuitously isolated along with the B's during the microdissection step.

Although shorter fixation times yield longer and more numerous microclones, we have found fixation times shorter than five minutes do not adequately fix meiotic chromosomes from these rye +B genotypes. The use of synchronized protoplasts to prepare chromosomes may provide cleaner preparations, but the stability and synchronization of such cultures in +B rye genotypes present many difficulties.

It may be possible to avoid the relatively inefficient microcloning steps by using PCR amplification to amplify microdissected chromosomes or chromosome fragments. In the case of the rye B-chromosome, we have a number of potential clones which would provide PCR primers. We are currently investigating the possibilities of performing PCR directly on microdissected B-chromosomes using primers to known B-chromosome clones, in order to test the efficiency of this process. Given success in this approach, we plan to use random nine-mer sequences as primers for PCR. Predictions of the distribution of such sequences suggest that, unless the B-chromosome shows very strong sequence composition bias, such primers should be capable of amplifying sequences from microdissected material.

Acknowledgments: This work was supported by research grants from the Science and Engineering Research Council and the Agriculture and Food Research Council of the United Kingdom.

References

Amos, A. and G. A. Dover. 1981. The distribution of repetitive DNAs between regular and supernumerary chromosomes in species of *Glossina* (Tsetse): a two step process in the origin of supernumeraries. Chromosoma 81:673-690.

Bates, G. P., B. J. Wainwright, R. Williamson, and S. D. M. Brown. 1986. Microdissection of and microcloning from the short arm of human chromosomes 2. Mol. Cell. Biol. 6:3826-3830.

Brown, S. D. M. and A. J. Greenfield. 1987. A model to describe the size distribution of mammalian genomic fragments recovered by microcloning. Gene 55:327-332.

Davis, L. G., M. D. Dibner, and J.F. Battey. 1986. Growth and preparation of bacteriophage. In *Basic Methods in Molecular Biology* (Davis, L.G., Dibner, M.D. and Battey, J.F. eds.) pp. 158-160. Elsevier.

Dover, G. A. 1975. The heterogeneity of B-chromosome DNA: no evidence for a B-chromosome specific repetitive DNA correlated with B-chromosome effects on meiotic pairing in *Triticinae*. Chromosoma 53:153-173.

Dover, G. A. 1976. Molecular properties of B-chromosome DNA: a clue to its origin in the *Triticinae* and *Orthoptera*. *Current Chromosome Research*. Jones, K. and Brandham, P.E. (eds.) Elsevier/North-Holland, Biomedical Press, Amsterdam.

Feinberg, A. P. and B. Vogelstein. 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266-267.

Fisher, E. M. C., J. S. Cavanna, and S. D. M. Brown. 1985. Microdissection and microcloning of the mouse X-chromosome. PNAS 82:5846-5849.

Jones, R. N. and H. R. Rees. 1982. *B-Chromosomes*. pp. 1-266. Academic Press.

Kishikawa, H. 1965. Cytogenetic studies of B-chromosomes in rye, *Secale cereale* L. in Japan. Agric. Bull. Saga Univ. 21:1-81.

Raeder, U. and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. Let. Appl. Microbiol. 1:17-21.

Reed, K. C. 1986a. Alkaline southern blotting to Zetaprobe membrane. Biorad Bulletin No. 1233.

Reed, K. C. 1986b. Nucleic acid hybridizations with DNA bound to Zetaprobe membrane. Biorad Bulletin No. 1234.

Rimpau, J. and R. B. Flavell. 1975. Characterization of rye B-chromosome DNA by DNA/DNA hybridization. Chromosoma 52:207-217.

Sandery, M. J., J. W. Forster, and R. N. Jones. 1989. Identification of a putative B-chromosome specific sequence from rye (*Secale cereale*). Heredity 62:282.

Scalenghe, F., E. Tusco, J. E. Edstrom, V. Pirotta, and M. L. Melli. 1981. Microdissection and cloning of DNA from a specific region of *Drosophila melanogaster* polytene chromosomes. Chromosoma 82: 205-216.

Timmis, J. N., J. Ingle, J. Sinclair, and R. N. Jones. 1975. The genomic quality of rye B-chromosomes. J. Exp. Bot. 26:367-378.

Young, R. A. and R. W. Davis. 1983. Efficient isolation of genes using antibody probes. PNAS 80:1194-1198.