Chromosomal localization of a tandemly repeated DNA sequence in *Trifolium repens* L.

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ABSTRACT

A karyotype of *Trifolium repens* constructed from mitotic cells revealed 13 pairs of metacentric and 3 pairs of submetacentric chromosomes including a pair of satellites located at the end of the short arm of chromosome 16. C-bands were identified around the centromeric regions of 8 pairs of chromosomes. A 350 bp tandemly repeated DNA sequence from *T. repens* labelled with digoxigenin hybridized to the proximal centromeric regions of 12 chromosome pairs. Some correlation between the distribution of the repeat sequence and the distribution of C-banding was demonstrated.

Key words: *Tandem repeat, in situ hybridization, digoxigenin-labelled probe, C-banding, karyotype.*

INTRODUCTION

Trifolium repens L, white clover, is an economically important plant species in temperate pastures. As briefly reported by [1], it has 16 pairs of chromosomes ($2n = 32$). As yet, no detailed cytological examination of this species, such as C-banding,

Chromosomal localization of a tandemly repeated DNA sequence in *T. repens*

has been reported. In the last decade, the technique of C-banding has been used to examine highly repeated sequences in plant chromosomes and has provided a useful tool for the analysis of cytogenetic structure in crop plants[2-7].

In plants, the chromosomal localization of highly repeated DNA sequences by *in situ* hybridization using non-radioactive labelling and detection techniques has been reported for cereals[8-12], *Allium*[13], tomato[14-16], rice[17], soybean[18], *Picea*[19], *Festuca*[20], and *Arabidopsis*[21].

For this study, a cytological examination of *T. repens* chromosomes from root tips was undertaken to obtain a basic karyogram using conventional Giemsa staining as well as more detailed C-banding information. In addition, a tandemly repeated, 350 bp, *T. repens* DNA sequence labelled with digoxigenin was used for an *in situ* hybridization analysis of *T. repens* chromosomes.

MATERIALS AND METHODS

Plant material and chromosome preparation

Seeds of *T. repens* cv. Huia were obtained from the Margot Forde Germplasm Centre (New Zealand Pastoral Agriculture Research Institute, Grasslands Research Centre, Palmerston North, New Zealand). Seeds were germinated at room temperature for 2-3 d in a moist petri dish. Root tips of 2-3 d old seedlings were treated with either a saturated solution of paradichlorobenzene (BDH) for 2-3 h or 0.05% colchicine (BDH) for 1-2 h and then fixed in a freshly prepared solution of 1:3 acetic acid/ethanol overnight at 4 °C. Chromosome preparations were carried out according to the procedure of Zhu and Gardiner[22]. Briefly, the enzyme treatment was used to weaken cell walls of root tips. Partially digested root tips were rinsed in distilled water and then placed in a drop of 45% acetic acid on a glass slide. The meristematic region of the root tip was excised teased out. Cells and chromosomes were gently squashed under a glass cover slip on a warm plate and stored at room temperature for use.

C-banding

C-banding was carried out according to the procedure of Zhu and Gardiner[23]. Slides were examined under a microscope (Zeiss) with a 100×oil objective. Mitotic spreads displaying sharp C-bands were photographed on Kodak Technique Pan black and white 2415 film.

DNA probe and in situ hybridization

Clone pTr350.1 (EMBL Accession No. X58701) containing a 350 bp tandemly repeated *T. repens* DNA sequence element was subcloned from a lambda genomic clone with an approx, 11kb insert consisting entirely of monomeric 350 bp and dimeric 700 bp repeating elements (data not shown). The insert from clone pTr350.1 was labelled with digoxigenin-11-dUTP using random primer labelling according to the manufacturer's specifications (Boehringer Mannheim).

In situ hybridization was carried out according to the procedure of Zhu and Gardiner[22]. Hybridization sites were detected by an alkaline phosphatase-conjugated antibody according to the manufacturer's instructions for the labelling and detection kit (Boehringer Mannheim). Visualization of the bound antibody was accomplished by an enzymatic colour reaction, the colourimetric substrates nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) producing a dark purple/brown precipitate at the sites of hybridization on the chromosome. Controls consisted of a chromosome spread pretreated with 50 µg/ml DNase (Sigma) at 37 °C for 30 min and a chromosome spread carried through the hybridization procedure but without hybridization of a probe.

Measurements were made from photographs of 11 mitotic spreads. Relative chromosome lengths and arm ratios were calculated according to Macgregor and Varley[24]. Karyotypes of chromosomes were made from enlarged photomicrographs with the chromosomes arranged in order of decreasing length. The chromosomes were then compared on the basis of centromere position, arm ratio, and the sizes of the satellites.

RESULTS AND DISCUSSION

Cytological analysis

T. repens has 16 pairs of chromosomes with a pair of satellites located at the end of the short arm of chromosome 16 (Fig 1), confirming the earlier results reported by Chen and Gibson[1]. A somatic chromosome karyotype was constructed from 11 mitotic cells by arranging the chromosome pairs on the basis of decreasing size and centromere position, with classification on the basis of the arm ratio using the criteria of Levan *et al.* [25] (Tab 1). Karyotype analysis showed that 13 pairs of chromosomes are metacentric (chromosomes 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, and 15) and 3 pairs are submetacentric (chromosomes 2, 13, and 16). In contrast, Chen and Gibson[1], in a less detailed analysis, reported that 4 pairs of the *T. repens* chromosomes were metacentric, 11 pairs were submetacentric, and 1 pair was telocentric.

Fig 1. Giemsa stained karyotype of chromosomes from root tip of *Trifolium repens*.

C-banding analysis

C-bands were observed at the proximal centromeric regions of chromosome pairs 2, 3, 5, 7, 9, 11, 13, and 15 while the other 8 pairs of chromosomes lacked C-bands (Fig 2). Neither terminal nor interstitial C-bands were observed. This C-banding pattern was also observed by Dr H. Ansari (personal communication). Giemsa C-banding is associated with highly repeated DNA sequences in constitutive hete-

Chromosomal localization of a tandemly repeated DNA sequence in *T. repens*

rochromatin[24]. The C-banding technique has been used to visualize the cytogenetic condition and the chromosomal composition of plants such as cereals[2, 4-6, 11, 26-27] and *Vicia* species[28].

Tab 1. Mean length, arm ratio, and centromere index values derived from eleven metaphase chromosomes of *Trifolium repens*.

Chromosome number	Relative length	Arm ratio	Centromere position
1	8.14	1.38	m
2	7.40	1.71	sm
3	7.10	1.59	m
4	6.82	1.50	m
5	6.76	1.59	m
6	6.49	1.66	m
7	6.40	1.61	m
8	6.28	1.41	m
9	6.15	1.67	m
10	6.05	1.61	m
11	5.94	1.67	m
12	5.81	1.65	m
13	5.59	1.72	sm
14	5.41	1.38	m
15	5.08	1.54	m
16	4.58	2.73	sm
sat.	1.15		

m=median region sm=submedian region sat.=satellite

Fig 2. Karyotype and corresponding mitotic spread of *Trifolium repens* chromosomes at metaphase.

(A) Giemsa staining. (B) C-banding.

In situ hybridization

The digoxigenin-labelled 350 bp *T. repens* repeat DNA hybridized to 12 pairs of chromosomes (chromosome pairs 1, 2, 3, 5, 6, 7, 9, 11, 12, 13, 15, and 16) at proximal centromeric regions (Fig 3) with a 90% frequency based on 11 karyotypes (data not shown). In all cases the hybridization signals were strong and could even be observed at late prophase (Fig 4). A low frequency of apparent hybridization (<27%) at the proximal centromeric regions of chromosomes 4, 8, 10, and 14 was observed. The hybridization signals on the small chromosomes of *T. repens* obtained using the digoxigenin-labelled probe were considerably more precise and distinct than those obtained using radioactively-labelled probes (data not shown).

Fig 3. Karyotype and corresponding mitotic spread of *Trifolium repens* chromosomes at early metaphase.

(A) Giemsa staining.

(B) *In situ* hybridization of digoxigenin-labelled 350 bp repeat DNA.

Three classes of tandemly repeated DNA sequences in human chromosomes have been identified, i.e. classical satellite, α -satellite, and β -satellite DNA[29]. All of these sequences are located in the variable heterochromatic regions on particular chromosomes[30]. The nucleotide sequence and the organization of monomeric units varies from one chromosome to another in the form of chromosome-specific, higher order, tandemly repeated arrays (alphoid subfamilies)[29].

Alphoid-like satellite DNA sequences have been characterised for a number of plant species including radish[31], cereals[9], and members of the Liliaceae[32]. In all cases these sequences were located in the variable heterochromatic regions of

Chromosomal localization of a tandemly repeated DNA sequence in *T. repens*

specific chromosomes. For *T. repens*, the 350 bp repeat DNA was found to be localized in heterochromatin around the centromeric regions on specific chromosomes and appeared to be one component of the centromeric DNA of this species. Chromosomes 4, 8, 10, and 14, which did not exhibit, or exhibit only a low frequency of hybridization with this probe, may have different and/or variable satellite DNA sequences involved in these centromeric regions as is the case in humans and cereals as discussed above. Alternatively, since *T. repens* is tetraploid, this may reflect differences within the genomes of the respective parental species.

Fig 4. As for Fig 3 but at late prophase.

For *T. repens*, the C-banding technique has revealed the presence of heterochromatin around the centromeric regions of some chromosomes. There is some similarity between the patterns of labelling revealed by *in situ* hybridization with the 350 bp repeat DNA and the C-banding pattern. Chromosome pairs 1, 6, and 13 (Fig 3) show obvious *in situ* hybridization signals where there are no apparent C-bands, probably reflecting the greater loss of some nucleic acid during C-banding procedure and reflecting the more sensitivity of the *in situ* hybridization technique. However, chromosome pairs 2, 7, and 9 all show clear hybridization of the 350 bp repeat DNA to both homologues of each pair of chromosomes yet only one of each of the homologues exhibits C-banding. This phenomenon needs to be further investigated.

Additional future work could involve an investigation into the distribution of the

350 bp repeat sequences of *T. repens* in closely related clover species. The use of fluorescent staining[33] and fluorescent *in situ* hybridization techniques using the 350 bp repeat sequence probe, along with other probes, could also be considered in order to analyse the chromosomal structure of *T. repens* in greater detail.

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