RAPD markers encoding retrotransposable elements are linked to the male sex in *Cannabis sativa* L.

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Abstract: Male-associated DNA sequences were analyzed in *Cannabis sativa* L. (hemp), a dioecious plant with heteromorphic sex chromosomes. DNA was isolated from male and female plants and subjected to random amplified polymorphic DNA analysis. Of 120 primers, 17 yielded 400 to 1500-bp fragments detectable in male, but not female, plants. These fragments were cloned and used as probes in gel-blot analysis of genomic DNA. When male and female DNA was hybridized with 2 of these male-specific fragments, MADC(male-associated DNA sequences in *C. sativa*)3 and MADC4, particularly intense bands specific to male plants were detected in addition to bands common to both sexes. The MADC3 and MADC4 sequences were shown to encode gag/pol polyproteins of *copia*-like retrotransposons. Fluorescence in situ hybridization with MADC3 and MADC4 as probes revealed a number of intense signals on the Y chromosome as well as dispersed signals on all chromosomes. The gel-blot analysis and fluorescence in situ hybridization results presented here support the hypothesis that accumulation of retrotransposable elements on the Y chromosome might be 1 cause of heteromorphism of sex chromosomes.

Key words: Cannabis sativa, FISH, RAPD, retrotransposon, sex chromosome.

Résumé : Des séquences d'ADN associées au sexe mâle ont été analysées chez le *Cannabis sativa* L. (chanvre), une plante dioïque avec des chromosomes sexuels hétéromorphes. L'ADN a été extrait de plantes mâles ou femelles et assujetti à une analyse RAPD (ADN polymorphe amplifié au hasard). Des 120 amorces employées, 17 ont produit des amplicons de 400 à 1500 pb présents chez les mâles mais absents chez les femelles. Ces amplicons ont été clonés et employés comme sondes lors d'hybridations Southern. Lorsque l'ADN mâle ou femelle a été hybridé à 2 de ces sondes mâle-spécifiques, MADC(« male-associated DNA sequences in *C. sativa* »)3 et MADC4, des bandes particulièrement intenses et mâle-spécifiques ont été détectées en plus de bandes communes aux 2 sexes. Les séquences MADC3 et MADC4 codent pour des polyprotéines gag/pol de rétrotransposons de type *copia*. Des hybridations in situ en fluorescence avec MADC3 et MADC4 ont révélé plusieurs signaux intenses sur le chromosome Y de même que des signaux dispersés sur tous les chromosomes. Les résultats de l'analyse Southern et de l'hybridation in situ supportent l'hypothèse voulant que l'accumulation de rétrotransposons sur le chromosome Y serait une cause de l'hétéromorphisme des chromosomes sexuels.

Mots clés : Cannabis sativa, FISH, RAPD, rétrotransposon, chromosomes sexuels.

[Traduit par la Rédaction]

Introduction

The majority of animal species are dioecious with separate male and female individuals, whereas the majority of flowering plant species are hermaphroditic with bisexual flowers. Nevertheless, approximately 6% of flowering plants are dioecious (Renner and Ricklefs 1995). Dioecious plants are thought to be the most evolved members of the plant

Received 19 May 2005. Accepted 24 May 2005. Published on the NRC Research Press Web site at http://genome.nrc.ca on 21 October 2005.

Corresponding Editor: J.H. de Jong.

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 doi: 10.1139/G05-056

kingdom in terms of sex differentiation, and heteromorphic sex chromosomes have been reported in a number of species such as *Silene latifolia* (*Melandrium album*), *Cannabis sativa* L. (hemp), *Humulus lupulus* L. (hop), and *Rumex acetosa* L. (sorrel) (Chattopadhyay and Sharma 1991).

The majority of known dioecious plants have a XX/XY chromosome arrangement similar to that in mammals, in which the structure and function of sex chromosomes have been well characterized. However, in contrast with mammals, the Y chromosome in *Silene*, *Rumex*, and *Cannabis* is much larger than the X chromosome. The mammalian Y chromosome has been shown to include male-determining genes such as *Sry* and male organ-forming genes (Sinclair et al. 1990; Reijo et al. 1995). In plants, however, the structure and origin of sex chromosomes as well as their role in sex determination are poorly understood, despite the number of studies involved with the roles of sex chromosomes in sex determination in haploid, diploid, and triploid plants (Warmke and Davidson 1944; Nishiyama et al. 1947).

The relationship between polyploidy and sex expression in flowers has been studied using mutants of *Silene*, a model plant used to study sex determination, and as a result, the male-determining region of the Y chromosome has been identified (Westergaard 1946, 1948; Grant et al. 1994). In *C. sativa*, another well-studied dioecious plant containing XX (female) and XY (male) genotypes, the X and Y chromosomes are known to carry female- and male-determining genes, respectively, whereas the autosomes are not involved in sex determination (Warmke and Davidson 1944). As mentioned earlier, the Y chromosome in *C. sativa* is much larger than both the X chromosome and autosomes (Yamada 1943; Sakamoto et al. 1998).

Random amplified polymorphic DNA (RAPD) is widely used in genetic mapping, detection of phenotypic variation, and evolutionary studies. It is also very useful in studies of sexual differentiation and identification of dioecious plants. Using this approach, researchers have identified molecular markers of female and male plants in various species such as *Silene* (Mulcahy et al. 1992; Zhang et al. 1998; Nakao et al. 2002), *Humulus lupulus* L. (Polly et al. 1997), *Pistacia vera* L. (Hormaza et al. 1994), and *Actinidia chinensis* (Harvey et al. 1997). Six male-specific RAPD markers, MADC(male-associated DNA sequences in *C. sativa*)1 to MADC6, have also been identified in *C. sativa* (Sakamoto et al. 1995; Mandolino et al. 1999; Torjek et al. 2002). In the present study, we characterize 2 of these RAPD markers, MADC3 and MADC4, and discuss the relationship between their sequences and the structure of the Y chromosome in *C. sativa*.

Materials and methods

Plant material

Seeds of *C. sativa* L. var. *sativa* (CBDA) obtained from random crossbreeding between males and females of various CBDA strains were used for plant cultivation. Plants were grown at 25 °C with a daily light-dark cycle of 16 h light and 8 h dark until they reached 50–100 cm in height, at which point they were exposed to 8 h light and 16 h dark to induce flowering. Plants were propagated vegetatively from cuttings. Five male and 5 female plants were used in this experiment.

Isolation of genomic DNA and RAPD analysis

Genomic DNA was isolated from young leaves of the inbred *C. sativa* plants using a Nucleon Phyto Pure Plant DNA Extraction Kit (Amersham Bioscience, Piscataway, New Jersy, USA). The reaction mixture (5 μL) for polymerase chain reaction (PCR) contained 5 ng of DNA in 1 μL of Tris–EDTA buffer, 0.4 μL of 1 mol Tris–HCl/L (pH 9.0), 0.2 μL of 0.5 mol $(NH_4)_2SO_4$/L, 0.2 μL of 2.5 mmol dATP/L, 0.2 μL of 2.5 mmol dCTP/L, 0.2 μL of 2.5 mmol dGTP/L, 0.2 μL of 2.5 mmol dTTP/L, 1 μL of 17.5 mmol $MgCl_2$/L, 0.2 μL of a 10 μmol primer solution /L, 1 μL of distilled water, and 0.2 μL of TaKaRa Ex *Taq*™ DNA Polymerase (5 U/μL; Takara Shuzo, Ootsu, Japan). After incubation at 92 °C for 3 min, 45 cycles of PCR were performed at 95 °C for 30 s, 34 °C for 15 s, 74 °C for 1 min, and a final incubation was performed at 72 °C for 8 min. Primers included 120 arbitrary oligonucleotides from Operon 10-mer primer kits A to F (Operon Technologies, Alameda, California, USA). PCR products were separated by 1.2% agarose gel electrophoresis and visualized after ethidium bromide staining.

Cloning and sequencing

Male-specific bands were cloned into a pCR II vector (Invitrogen, San Diego, California, USA). Then, nucleotide sequences were determined using the dye deoxy chain-termination method with a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, New Jersey, USA) and DNA sequencer (model ABI PRISM 377 or 310; Perkin-Elmer). The Advanced BLAST search (BLAST version 2.2.6) of the National Center for Biotechnology Information (NCBI) was used to perform homology searches.

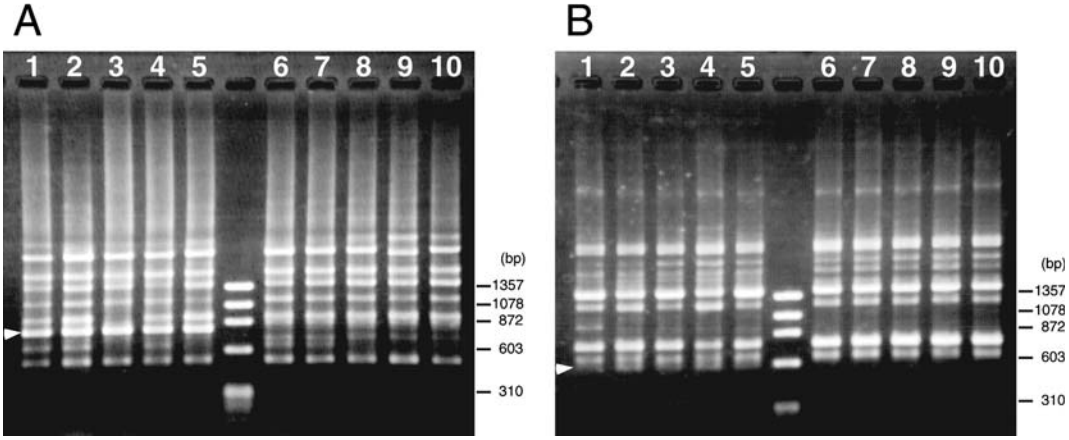
Gel-blot analysis of genomic DNA

Quantities of DNA (10 μg) isolated from young leaves of individual male and female plants were separately digested with restriction endonucleases (*Eco*RI, *Bam*HI, and *Hin*dIII). The digests were fractionated onto a 1.0% (*w/v*) agarose gel and transferred onto a GeneScreen Plus membrane (Du Pont, Boston, Massachusetts, USA) and then hybridized with MADC3 and MADC4 labeled with (α-^{32}P) dCTP using a Ready-To-Go™ DNA Labeling Beads (-dCTP) kit (Amersham Biosciences, Amersham, UK) as probes. Membranes were exposed to Hyperfilm™ MP film (Amersham Biosciences, Amersham, UK).

Preparation of chromosomes

Root tips approximately 5 mm in length were excised and incubated in distilled water at 11 °C for 12–14 h and then fixed in a mixture of ethanol and acetic acid (1:1 *v/v*). Samples for examination of chromosomes were prepared according to the enzymatic maceration/air-drying method (Ohmido and Fukui 1996, 1997). Briefly, root tips were macerated in a cocktail of enzymes (2% Cellulase Onozuka RS (Yakult Farma, Tokyo, Japan), 1.5% Pectolyase Y-23 (Seishin Farma, Tokyo, Japan), 0.3% Macerozyme R200 (Yakult Farma), and 0.5 mmol EDTA/L, pH 4.2) at 37 °C for 30–40 min and then spread on glass slides and air-dried.

Fig. 1. Patterns produced by PCR. The DNA from 5 male (1–5) and 5 female (6–10) plants was used as templates. The patterns shown here were obtained using OPB18 (A) and OPC4 (B) as primers. Positions and sizes of markers are indicated on the right. Arrowheads indicate bands specific to male plants.



Fluorescence in situ hybridization (FISH)

FISH was performed as described by Sakamoto et al. (2000) and Ohmido et al. (1998, 2000). MADC3 and MADC4 were labeled with biotin-16-dUTP by PCR. Then, a hybridization mixture containing 5 ng/μL biotin-labeled probes, 50% formamide, 2× SSC, and 500 ng/μL sheared salmon sperm DNA was prepared. After overnight hybridization, the chromosome spreads were washed twice in 2× SSC (0.15 mol NaCl/L plus 0.015 mol sodium citrate/L), twice in 50% formamide/2× SSC, twice in 2× SSC at 42 °C, twice in 2× SSC at room temperature and were equilibrated in 4 × SSCT (4 × SSC with 0.2% Tween 20). Probes were detected by labeling with fluorescein-conjugated avidin DCS (Vector Laboratories, Burlingame, California, USA) followed by biotinylated antibodies against avidin D (Vector Laboratories) in l% nonfat milk in 4× SSCT. Blocking was performed with blocking solution (5% nonfat milk or 5% goat serum) at 37 °C for 60 min and then with fluorescein-conjugated avidin DCS (Vector Laboratories). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride in Vectashield® mounting medium (Vector Laboratories) before analysis. Green fluorescein isothiocyanate fluorescent signals and the blue fluorescence of the chromosome counterstain were detected using a fluorescence microscope equipped with appropriate filters. The 2 images were captured separately with a cooled CCD camera (PXLl400, Photometrics, Tucson, Arizona, USA) and merged into single images with IP-Lab (Photometrics, Tucson, Arizona, USA) and Photoshop® imaging software (Adobe Systems, San Jose, California, USA).

Results

Comparison of male and female DNA using RAPD analysis

Of 120 primers, 17 yielded 400 to 1500-bp fragments specific to male plants. All male-specific DNA fragments were cloned and used as probes in gel-blot analysis of genomic DNA. With 11 of these 17 male-specific probes, there were no differences in hybridization patterns between DNA from male and female plants. In contrast, when male and female DNA was hybridized with the remaining 6 probes (OPB-9, OPB-12, OPB-18, OPC-4, OPE-20, and OPF-3), bands specific to the male plants only were detected in addition to bands common to DNA from both sexes. Two fragments in particular (MADC3 and MADC4 from OPB-18 and OPC-4, respectively) gave unusually intense male-specific bands. Figure 1 shows the PCR products obtained with the OPB-18 and OPC-4 primers. As shown in Fig. 2A, when DNA was hybridized with MADC3, strong male-specific bands were clearly detected with *Eco*RI and *Hin*dIII digestion, whereas with the MADC4 probe strong male-specific bands were detected when digested with *Eco*RI (Fig. 2B). In addition, the signals from male DNA digested with *Bam*HI were stronger than those of female DNA (Fig. 2B). Identical results were obtained in the analysis of various CBDA strains (data not shown).

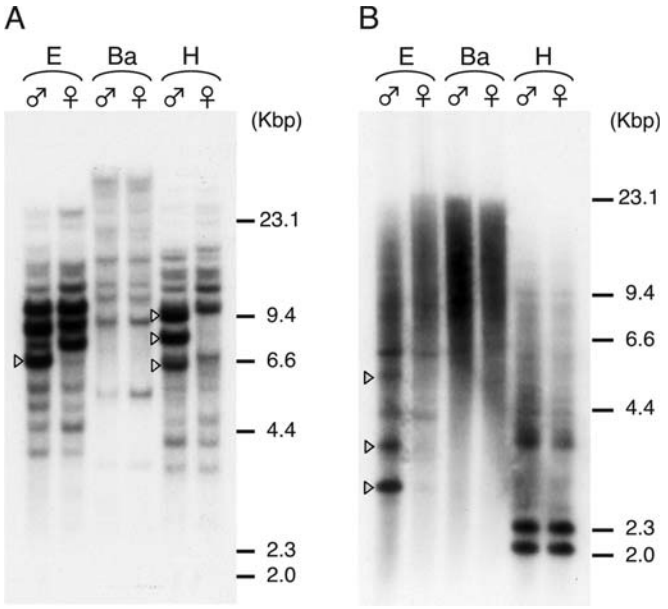
Characterization of the MADC3 and MADC4 sequences

The sequences of 2 of the male-specific fragments, MADC3 and MADC4 (GenBank accession Nos. AB021658 and AB021659, respectively), were determined in this study. Analysis of the 771-bp long MADC3 sequence revealed the presence of a coding region highly homologous to open reading frames encoding the gag/pol polyprotein of *copia*-like retrotransposons in various plant species, such as *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, and *Glycine max*. A fragment approximately 540 bp long at the 5′ end of the element was homologous to RNAse H in the polyprotein of the *copia*-like retrotransposons. On the other hand, the 576-bp long MADC4 was similar to retrotransposable elements from *A. thaliana* and *Oryza* at the amino acid level. An approximately 160-bp fragment at the 5′ end of MADC4 shared 60% amino acid similarity with the integrase in the polyprotein of the *copia*-like retrotransposon from *Oryza australiensis*, RIRE1 (accession No. BAA22288.1). The general structure of the *copia*-like retrotransposon and positions of MADC3 and MADC4 are shown in Fig. 3.

Fluorescence in situ hybridization

To examine the chromosomal localization of MADC3 and MADC4, metaphase chromosomes from root tips of *C. sativa* were subjected to FISH using biotin-labeled fragments as

Fig. 2. Gel-blot analysis of genomic DNA probed with MADC3 (A) and MADC4 (B). DNA from male (♂) and female (♀) plants was separately digested with *Eco*RI (E), *Bam*HI (Ba), and *Hin*dIII (H). Fragments were separated on gel, transferred to a membrane, and probed with either MADC3 or MADC4. Positions and sizes of markers are indicated on the right. Arrowheads indicate intense bands specific to male DNA.

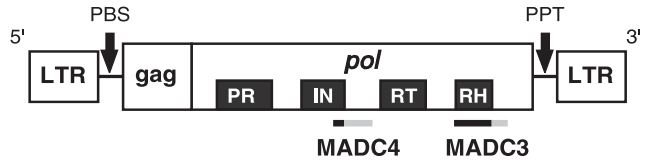


probes. Using MADC3 and MADC4 as probes, sporadic signals were detected on the Y chromosome, the largest chromosome in *C. sativa*, in addition to dispersed signals on all chromosomes (Figs. 4A and 4B). Moreover, when MADC4 was used as the probe, numerous intense signals were detected on 1 set of autosomes (Figs. 4C and 4D). No Y chromosome-specific signals were detected with either MADC3 or MADC4.

Discussion

In our previous paper, the male-associated DNA sequence MADC1 was cloned in *C. sativa* L. using a RAPD technique (Sakamoto et al. 1995); MADC1 was the most conspicuous DNA fragment giving a male-specific pattern in gel-blot analysis. In particular, *Hin*dIII digestion yielded 2 intense bands detectable only in male plants. Other RAPD markers specific to male *C. sativa* plants have also been reported. Mandolino et al. (1999) cloned MADC2 in *C. sativa* cultivars using the 10-meric primer OPA08, and Torjek et al. (2002) isolated MADC5 and MADC6 using the 10-meric primers UBC354 and OPD05, respectively. Further studies using these RAPD markers will likely be useful in constructing deletion and linkage maps of the Y chromosome and determining the Y-chromosome fraction that does not recombine with the X chromosome. In this study, gel-blot analysis of MADC3 and MADC4 revealed multiple bands, only some of which showed sex differences. Whereas some bands specific to male plant DNA seem to be linked to the Y chromosome, similar multiple sequences for retrotransposons, for example, able to hybridize with MADC3 and MADC4, are thought to exist on the *C. sativa* genome.

Fig. 3. General structure of *copia*-type retrotransposon and the positions of the fragments encoded by MADC3 and MADC4. LTR, gag, and *pol* indicate the long terminal repeat, gag protein, and polyprotein, respectively. PR, IN, RT, and RH show the conserved amino acid domains of protease, integrase, reverse transcriptase, and RNAse H, respectively. Black areas within the black and gray bars indicate the regions homologous among the *copia*-type retrotransposon, MADC3 and MADC4.

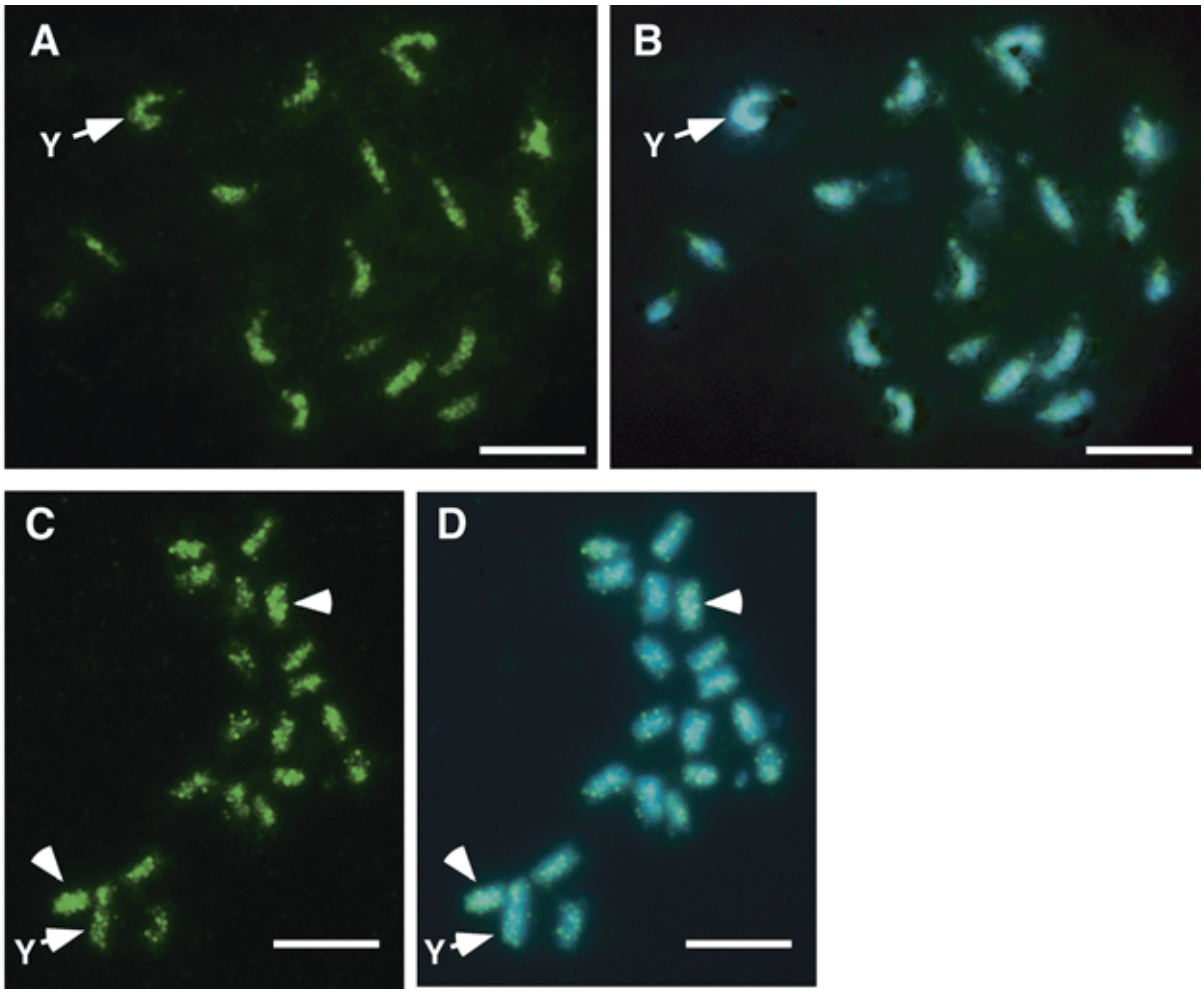


Retrotransposons, as with retroviruses, are transposed by reverse transcription of an RNA intermediate. They can be classified into two groups based on whether they have long terminal repeats (LTRs) (Boeke and Corres 1989). NonLTR retrotransposons can be further divided into long and short interspersed elements (LINEs and SINEs, respectively) (Boeke 1989), whereas LTR retrotransposons can be subdivided into Ty1-*copia* and Ty3-*gypsy* based on the arrangement of the protein. The structure of the polyprotein in Ty1-*copia* type retrotransposons from the 5′ to the 3′ end consists of protease, integrase, reverse transcriptase, and RNAse H. This present study revealed, for the 1st time, that MADC3 and MADC4 both encode fragments of this polyprotein.

We previously reported that the nuclear DNA content of male *C. sativa* plants is 47 Mbp larger than that of female plants owing to the large Y chromosome (Sakamoto et al. 1998). In addition, the Y chromosome was shown to be strongly stained by Giemsa and DAPI (Sakamoto et al. 1998) suggesting that the Y chromosome has abundant heterochromatic regions including tandem and dispersed repetitive sequences such as transposable elements. The majority of the identified sex-related RAPD markers in *C. sativa*, therefore, seem to encode unknown or retrotransposon-like sequences (Mandolino et al. 1999; Sakamoto et al. 2000; Torjek et al. 2002). In addition to RAPD markers, however, other markers exist such as AFLP markers, which are also linked to sex chromosomes (Peil et al. 2003) and are therefore potentially useful in obtaining insight into the structure of the Y chromosome.

In our previous study, LINE-CS (LINE-like retrotransposon in *C. sativa*), which gave male-specific patterns in gel-blot analysis, accumulated at the terminal region of the long arm of the Y chromosome in *C. sativa* (Sakamoto et al. 2000). In this present study, however, FISH using MADC3 and MADC4 as probes detected numerous intense signals on the Y chromosome as well as dispersed signals on all chromosomes, and there were no extensive differences between the *copia*-like retrotransposon distribution on the Y chromosome and other chromosomes (Fig. 4). MADC4, however, produced intense signals on 1 pair of autosomes and is therefore also a marker for this set of small autosomes. In the dioecious plant *S. latifolia*, the Ty1-*copia* and Ty3-*gypsy* type retrotransposons are not sex-linked (Matsunaga et al. 2002), whereas in *Drosophila melanogaster*, FISH analysis revealed that the *copia*-type retrotransposon is more abundant in the Y chromosome compared with the abundance in

Fig. 4. Results of FISH using MADC3 and MADC4 with metaphase chromosomes from male root-tip cells. The green fluorescence in (A) and (C) indicates biotin-labeled MADC3 and MADC4 probes, respectively. In (B) and (D), the fluorescent green signals are merged with the blue fluorescence of the DAPI-stained chromosomes. Y indicates the Y chromosome. Arrowheads indicate a set of autosomes. Bars represent 5 μm.



the X chromosome and autosomes (Pimpinelli et al. 1995). In gel-blot analysis of genomic DNA using a *copia*-type retrotransposon as a probe, Junakovic et al. (1998) detected several male-specific fragments corresponding to clusters on the Y chromosome of *D. melanogaster* and *Drosophila simulans*. We therefore suggest that the relationship between the gel-blot patterns of genomic DNA and localizations on sex chromosomes are as follows. (*i*) Bands specific to male DNA are thought to localize on the Y chromosome; for example, as with the LINE-like retrotransposon in *C. sativa* and *copia*-like retrotransposon in *Drosophila*. (*ii*) Bands common to both sexes probably localize on autosomes or pseudoautosomal regions of the sex chromosomes. (*iii*) Bands stronger in female DNA are thought to localize on the X chromosome in XY types.

The Y chromosome-specific RAPD markers characterized here (MADC3 and MADC4) produced male-specific bands in gel-blot analysis, although FISH revealed no Y chromosome-specific signals with either marker. The reason for these results might be that multiple sequences encoding retrotransposable elements also exist ubiquitously in the genome of *C. sativa*. This study together with further investigations into the molecular structure of the Y chromosome will help elucidate the mechanisms of sex differentiation in plants.

Acknowledgements

This work was supported by the Sasakawa Scientific Research Grant from The Japan Science Society, and by The Sumitomo Foundation.

References

Boeke, J.D. 1989. Transposable elements in *Saccharomyces cerevisiae*. *In* Mobile DNA. *Edited by* D.E. Berg and M.M. Howe. American Society for Microbiology, Washington, D.C. U.S.A. pp. 594–617.

Boeke, J.D., and Corres, V.G. 1989. Transcription and reverse transcription of retrotransposons. Annu. Rev. Microbiol. **43**: 403–434.

Chattopadhyay, D., and Sharma, A.K. 1991. Sex determination in dioecious species of plants. Fed. Proc. **102**: 29–55.

Grant, S., Houben, A., Vyskot, B., Siroky, J., Pan, W.-H., Macas, J., and Saedler, H. 1994. Genetics of sex determination in flowering plants. Dev. Genet. **15**: 214–230.

Harvey, C.F., Gill, G.P., Fraser, L.G., and McNeilage, M.A. 1997. Sex determination in *Actinidia*. 1. Sex-linked markers and progeny sex ratio in diploid *A. chinensis*. Sex. Plant Reprod. **10**: 149–154.

Hormaza, J.I., Dollo, L., and Polito, V.S. 1994. Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. Theor. Appl. Genet. **89**: 9–13.

Junakovic, N., Terrinoni, A., Di, F.C., Vieira, C., and Loevenbruck, C. 1998. Accumulation of transposable elements in the heterochromatin and on the Y Chromosome of *Drosophila simulans* and *Drosophila melanogaster.* J. Mol. Evol. **46**: 661–68.

Mandolino, G., Carboni, A, Forapani, S., Faeti, V., and Ranalli, P. 1999. Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.). Theor. Appl. Genet. **98**: 86–92.

Matsunaga, S., Yagisawa, F., Yamamoto, M., Uchida, W., Nakao, S., and Kawano, S. 2002. LTR retrotransposons in the dioecious plant *Silene latifolia*. Genome, **45**: 745–751.

Mulcahy, D.L., Weeden, N.F., Kesseli, R., and Carroll, S.B. 1992. DNA probes for the Y-chromosome of *Silene latifolia*, a dioecious angiosperm. Sex. Plant Reprod. **5**: 86–88.

Nishiyama, I., Yamada, I., and Mazaki, M. 1947. Studies on artificial polyploid plants. XI. Changes of the sex ratio in the progeny of the autotetraploid hemp. Seiken Ziho, **3**: 144–151.

Ohmido, N., and Fukui, K. 1996. A new manual for fluorescence in situ hybridization (FISH) in plant chromosomes. Rice Genet. Newsl. **13**: 89–93.

Ohmido, N., and Fukui, K. 1997. Visual verification of close disposition between a rice and a genome-specific DNA sequence (TrsA) and the telomere sequence. Plant Mol. Biol. **35**: 963–968.

Ohmido, N., Akiyama, Y., and Fukui, K. 1998. Physical mapping of unique nucleotide sequences on identified rice chromosomes. Plant Mol. Biol. **38**: 1043–1052.

Ohmido, N., Kijima, K., Akiyama, Y., de Jong, J.H., and Fukui, K. 2000. Quantification of total genomic DNA and selected repetitive sequences reveals concurrent changes in different DNA families in indica and japonica rice. Mol. Gen. Genet. **263**: 388–394.

Peil, A., Flachowsky, H., Schumann, E., and Weber, W.E. 2003. Sex-linked AFLP markers indicate a pseudoautosomal region in hemp (*Cannabis sativa*) L. Theor. Appl. Genet. **107**: 102–109.

Pimpinelli, S., Berloco, M., Fanti, L., Dimitri, P., Bonaccorsi, S., Marcherri, E., Caizzi, E., Caggese, C., and Gatti, M. 1995. Transposable elements are stable structural components of *Drosophila melanogaster* heterochromatin. Proc. Natl. Acad. Sci. U.S.A. **92**: 3804–3808.

Polley, A., Seigner, E., and Ganal, M.G. 1997. Identification of sex in hop (*Humulus lupulus*) using molecular markers. Genome, **40**: 357–361.

Reijo, R., Lee, T., Salo, P., Alagappan, R., Brown, L., Rosenberg, M., Affe, T., Straus, D., Hovatta, O., Delachapelle, A., Silber, S., and Page, D. 1995. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat. Genet. **10**: 383–393.

Renner, S.S., and Ricklefs, R.E. 1995. Dioecy and its correlates in the flowering plants. Am. J. Bot. **82**: 596–606.

Sakamoto, K., Shimomura, K., Komeda, Y., Kamada, H., and Satoh, S. 1995. A male-associated DNA sequence in a dioecious plant, *Cannabis sativa* L. Plant Cell Physiol. **36**: 1549–1554.

Sakamoto, K., Akiyama, Y., Fukui, K., Kamada, H., and Satoh, S. 1998. Characterization: genome sizes and morphology of sex chromosome in hemp (*Cannabis sativa* L.). Cytologia, **63**: 459–464.

Sakamoto, K., Ohmido, N., Fukui, K., Kamada, H., and Satoh, S. 2000. Site-specific accumulation of a LINE-like retrotransposon in a sex chromosome of the dioecious plant *Cannabis sativa.* Plant Mol. Biol. **44**: 723–732.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., and Goodfellow, P.N. 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature (London), **346**: 240–244.

Torjek, O., Bucherna, N., Kiss, E., Homoki, H., Korpelova, F.Z., Bocsa, I., Nagy, I., and Heszky, L. 2002. Novel male-specific molecular markers (MADC5, MADC6) in hemp. Euphytica, **127**: 209–218.

Warmke, H.E., and Davidson, H. 1944. Polyploid investigation. Yearb. Carnegie Institution of Washington, **43**: 135–139.

Westergaard, M. 1946. Aberrant Y chromosomes and sex expression in *Melandrium album*. Hereditas, **32**: 419–431.

Westergaard, M. 1948. The relation between chromosome constitution and sex in the offspring of triploid *Melandrium*. Hereditas, **34**: 257–279.

Yamada, I. 1943. The sex-chromosome of *Cannabis sativa* L. Rep. Kihara Inst. Biol. Res. **2**: 64–68.

Zhang, Y.H., Di Stilio, V.S., Rehman, F., Avery, A., Mulcahy, D., and Kesseli, R. 1998. Y chromosome specific markers and the evolution of dioecy in the genus *Silene*. Genome, **41**: 141–147.