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In situ hybridization with species-specific DNA probes gives evidence for asymmetric nature of *Brassica* hybrids obtained by X-ray fusion

K. Itoh, M. Iwabuchi and K. Shimamoto *

Plantech Research Institute, 1000 Kamoshida, Midori-ku, Yokohama 227, Japan

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Summary. We have previously reported production of somatic hybrids between *B. oleracea* and *B. campestris* by fusion of *B. oleracea* protoplasts with X-irradiated *B. campestris* protoplasts, in order to transfer a part of the *B. campestris* genome into *B. oleracea*. Our previous analysis of morphology, chromosome number, and isozyme patterns of the hybrids suggested that they are asymmetric in nature. To obtain further evidence for the asymmetric nature of the hybrids, we isolated *B. campestris*-specific repetitive sequences and used them for in situ hybridization of the chromosomes of the hybrids. The repetitive DNA probes could specifically identify 8 out of 20 chromosomes of the *B. campestris* genome, and analysis of the hybrids indicates that 1–3 chromosomes of *B. campestris* are lacking in all five hybrids examined, giving clear evidence for the asymmetric nature of the hybrids. Furthermore, in situ hybridization revealed that some of the abnormal chromosomes observed in the hybrids are generated by rearrangements of *B. campestris* chromosomes caused by X-irradiation. Altogether, our study indicates that in situ hybridization using species-specific repetitive sequences is a useful tool to analyze chromosomal compositions of various types of hybrids obtained by cell fusion or conventional methods.

Key words: *Brassica* – Somatic hybrid – Repetitive sequence – In situ hybridization

Introduction

Production of somatic hybrids has been described in a number of species (for review, see Schieder and Vasil 1980; Schieder 1982). A detailed analysis of their chromosomal compositions, however, has not been extensively performed except in a few instances (Kao 1977; Hoffman and Adachi 1981). Possible reasons for the lack of such studies are the following. First, most of the somatic hybrids described are those in the Solanaceae, whose chromosomes are generally small and similar in morphology. Second, banding methods, which are well established in cereal species (Endo and Gill 1984) that contain relatively large chromosomes, have not been developed for the species containing smaller chromosomes. Third, when structural changes of chromosomes that are often observed by irradiation of protoplasts prior to cell fusion take place it becomes more difficult to analyze the chromosomes of the hybrids. For the above-mentioned reasons, chromosome studies of somatic hybrids have been generally limited to the analysis of chromosome number and certain chromosomes that have morphological characteristics, such as secondary constriction or satellites.

In situ hybridization of nucleic acid probes to metaphase chromosomes was originally developed in mammalian systems (Gall and Pardue 1969; Henderson 1982). In higher plants, this technique has been mainly used to study the organization of highly repetitive sequences in wheat (Hutchinson and Londsdale 1982), rye (Jones and Flavell 1982), *Allium* (Jamieson et al. 1986), and potato (Visser et al. 1988). More recently this technique has been applied for middle repetitive sequences (Ganal et al. 1988) and even for single- or low-copy sequences (Ambros et al. 1986a, b; Mouras et al. 1987; Huang et al. 1988).

Species-specific repetitive sequence has been shown to be a useful tool to analyze the nuclear genome of hybrids produced by cell fusion (Saul and Potrykus 1984). Using such sequences, the relative contribution of each parent's genome in asymmetric hybrids has been quantified (Imammura et al. 1987). In no cases, however,

* To whom correspondence and offprint requests should be addressed

have such species-specific DNA probes been used for the analysis of hybrids at the chromosome level.

We have previously described somatic hybrids of *B. oleracea* and *B. campestris* (Terada et al. 1987). More recently we have characterized somatic hybrids between these two species which were produced by X-irradiating *B. campestris* protoplasts prior to cell fusion (Yamashita et al. 1989). Analysis of their morphology, isozymes, and chromosome number suggested that most of the hybrids obtained in this experiment are asymmetric in nature, resulting from elimination of *B. campestris* chromosomes by X-ray irradiation. In this communication we describe further analysis of these asymmetric somatic hybrids of *B. oleracea* and *B. campestris*, by using in situ hybridization of *B. campestris*-specific, middle repetitive DNA sequences to metaphase chromosomes of the hybrids. Our analysis demonstrates the asymmetric nature of the hybrids obtained by X-ray fusion. Furthermore, in situ hybridization detected chromosomal rearrangements involving *B. campestris* chromosomes that are generated by irradiation of the protoplasts by X-ray prior to cell fusion.

Materials and methods

Plant materials

Somatic hybrid plants obtained by fusion of *B. oleracea* protoplasts with X-irradiated *B. campestris* protoplasts have been described previously (Yamashita et al. 1989). Their morphology, chromosome number, and isozyme patterns have been characterized. As control plants, *B. oleracea* var. *capitata* cv Nakarubi (cabbage), *B. campestris* var. *rapa* cv 77b (turnip), and var. *pekinensis* cv CR-strong (Chinese cabbage) were used.

Isolation of plant DNA

Nuclear DNA was isolated from leaves according to a published protocol (Murray and Thompson 1980) and purified by CsCl/EtBr density gradient centrifugation (Maniatis et al. 1982).

Isolation of repetitive sequences from B. campestris

B. campestris nuclear DNA digested with *Sal*I or *Taq*I was cloned at the *Sal*I or *Acc*I site of pUC19 by a standard method. *B. campestris*-specific repetitive sequences were screened by colony hybridization using total genomic DNAs of *B. campestris* and *B. oleracea* as probes.

Southern blot hybridization

Nuclear DNA digested with restriction enzymes was separated by electrophoresis and blotted to a nylon membrane according to Reed and Man (1985). Hybridization was performed in a solution containing 50% formamide, 5 × SSCP (1 × SSCP = 50 m*M* sodium phosphate, pH 6.8, 120 m*M* NaCl, 15 m*M* sodium citrate), 100 μg/ml carrier DNA, 0.5% nonfat milk, 10% dextran sulfate (w/v) for 16 h at 42 °C. After hybridization, the membrane was washed three times in 2 × SSC/0.1% SDS at 42 °C for 15 min and twice in 0.1 × SSC/0.1% SDS for 30 min at 65 °C.

Chromosome preparation

Plant material. Seedling roots of *B. oleracea* and *B. campestris* and root tips collected from hybrid plants grown in pots were used for chromosome preparation.

Pretreatment and fixation. Root tips (ca. 1 mm) were treated with a solution containing 0.02% colchicin and 7 μg/ml ethidium bromide for 3.75 h at 10 °C, and the solution was replaced with ice-cold Farmer's solution (3:1 = ethanol:glacial acetic acid) and kept overnight at −20 °C.

Refixation. After removing Farmer's solution by a Pasteur pipette, root tips were washed with distilled water for 2–3 min. After three washes the tissue was treated with an enzyme solution containing 4% cellulase "Onozuka" RS (Yakult), 1% pectolyase Y-23 (Seishin Pharmaceutical Co.), 75 m*M* KCl, and 7.5 m*M* EDTA (Nishibayashi and Kaeriyama 1986) for 45 min at 37 °C. Dispersed root-tip cells were then washed with 0.05 *N* NaOH overnight for 1 day and spread on a slide with forceps. Drops of Farmer's solution were occasionally added onto the slide to avoid desiccation of the cells. After air drying, the slides were kept for at least 2 week in a desiccator at room temperature before use for in situ hybridization. Approximately 50 slides were prepared for one experiment.

In situ hybridization

Denaturation of chromosomal DNA. RNase solution (100 μg/ml RNaseA in 2 × SSC) was dropped (100 μl/slide) on a slide with dispersed root-tip cells, and a coverslip was placed on the slide and kept in a humid chamber for 1 h at 37 °C. Then the slide was washed twice in 2 × SSC solution for 5 min at room temperature, and chromosome preparations were dehydrated in an ethanol series (70%, 90%, and 100%) for 10 min each and air dried for at least 15 min. For denaturation of chromosomal DNA, the slides were placed in 70% (v/v) formamide in 2 × SSC at 70 °C. To determine an optimal length of time for DNA denaturation, several test slides were denatured for 1–4 min and then examined for the morphology of chromosomes. Immediately after denaturation of chromosomal DNA, chromosomal preparations were dehydrated in an ice-cold ethanol series (70%, 90%, 100%) and air dried for more than 15 min.

Purification of probe DNA. Probe DNA was separated by electrophoresis, electroeluted and further purified by NenSorb 20 (Du Pont). Purified DNA was labeled with biotin-11-dUTP and biotin-7-dATP using a random prime labeling system (Amersham).

Hybridization. A solution containing biotin-labeled probe DNA (4 ng/μl biotinylated DNA, 50% formamide, 10% dextran sulfate, 0.6 μg/μl carrier DNA in 2 × SSC) was dropped onto a slide (50 μl/slide) and a siliconized coverslip was placed on it. Hybridization was performed for 16–18 h at 37 °C, and the slide was washed as described by Rayburn and Gill (1985).

Detection of hybridization signals. Hybridization signals were detected by streptavidin-alkaline phosphatase conjugate by using NBT-BCIP as substrates (BluGene, BRL). A solution containing 1 μg/ml streptavidin-alkaline phosphatase in Buffer 1, as described in the protocol supplied by the manufacturer (150 m*M* NaCl, 100 m*M* Tris-HCl at pH 7.5), was dropped on the slides (150 μl/slide) and kept at room temperature for 10 min. Then the slides were washed twice in Buffer 1 for 15 min and washed in Buffer 3 once (50 m*M* $MgCl_2$, 100 m*M* NaCl, 100 m*M* Tris-HCl, pH 9.5) for 10 min. After the wash, 100 μl of a reaction solution containing the substrates was added onto the slide. After 1-hour incubation at room temperature, the slide was washed with Buffer 3, covered with a cover glass and observed by a phase contrast microscope (Nikon Optiphoto). For photographing, Fujichrome DX100 and Minicopy HRII were used.

Results

Isolation of B. campestris-specific repetitive sequences

Two clones of middle repetitive sequences, CS1 and CT10, isolated from *B. campestris* were used for analysis of metaphase chromosomes of the somatic hybrids. A detailed characterization of these *B. campestris*-specific repetitive sequences will be described in an accompanying paper (Iwabuchi et al. 1991). CS1 is completely specific to the *B. campestris* genome and its copy number was estimated to be ca. 1,700 copies per haploid genome. The sequence CT10 is a variant form of CS1 consisting of CS1 and additional sequences that are hybridized with *B. oleracea* genome.

Southern blot analysis of the hybrids using a B. campestris-specific repetitive sequence as a probe

Southern blot analysis of three somatic hybrids whose chromosome compositions are distinct from one another was performed, in order to examine whether this repetitive sequence can be used to distinguish those hybrids presumably different in degree of asymmetry in the nuclear genome (Fig. 1). Based on our previous study on various characters of the hybrids, we concluded that these hybrids possess two times the *B. oleracea* genome, namely, 36 chromosomes, plus *B. campestris* chromosomes, whose number varies in each hybrid due to chromosome elimination caused by X-irradiation (Yamashita et al. 1989). The hybrids H15 (lane 2) and H13 (lane 3) contain 43 and 47 chromosomes, respectively, and the number of *B. campestris* chromosomes eliminated is 13 and 9, respectively. One hybrid, SH1, which was obtained through a standard cell fusion without X-irradiation and presumably contained a complete diploid genome of *B. campestris* (Y. Yamashita, R. Terada, K. Itoh, K. Shimamoto, unpublished results) was also examined (lane 4) as a control. In agreement with the assumption, the intensity of the hybridization signal increased in the order of H15, H13, and SH1. Because the DNA amount loaded in each lane was equal, this result suggested that the signal intensity in the hybrid is correlated with the number of *B. campestris* chromosomes present in the hybrid genome. Therefore, Southern blot analysis using the *B. campestris*-specific repetitive sequence as a probe supports our previous conclusion that the hybrids produced after X-irradiation of *B. campestris* protoplasts are asymmetric in nature.

Analysis of the hybrids by in situ hybridization

For analysis of the somatic hybrids, CS1 and a mixture of CS1 and CT10 were used as hybridization probes. First, *B. campestris* chromosomes were examined with two kinds of DNA probes (Table 1; Fig. 2a, c). The cloned DNA in the plasmid pCS1 clearly identified six chromosomes, whereas when a mixed probe, CS1 with CT10, was used, eight chromosomes of *B. campestris* were detected. Although the sequence containing CT10 was also present in the *B. oleracea* genome and a reduced amount of probe DNA (ca. ⅓ of the normal amount) was mixed with CS1, no detectable signals were observed in *B. oleracea* chromosomes (Fig. 2b). These control ex-

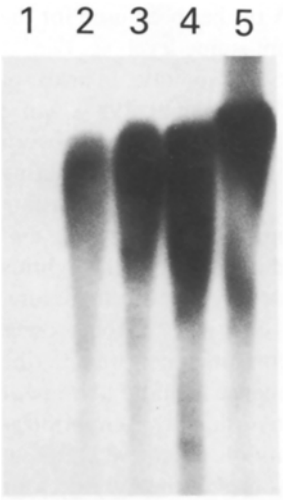

Fig. 1. Southern blot analysis of *Brassica* somatic hybrids and their parents by a *B. campestris*-specific DNA probe. Nuclear plant DNA was digested with *Taq*I, separated by electrophoresis, transferred to a nylon membrane, and hybridized with CS1. Preparation of the probe DNA and hybridization were described in "Materials and methods". *Lane 1: B. oleracea; lane 2:* hybrid H15; *lane 3:* hybrid H13; *lane 4:* hybrid SH1; *lane 5: B. campestris*

Table 1. Summary of in situ hybridization of *Brassica* hybrids and their parents

Plant	No. chromosomes (2n)	No. chromosomes hybridized with	
		CS1	CS1 + CS10
B. campestris	20	6	8
B. oleracea	18	0	0
H7	56	11	NT
H8	45	NT	5
H12	46	NT	6
H13	47	NT	7
H19	48	4	6

All the hybrids are assumed to contain 2 × *B. oleracea* genome, namely, 36 chromosomes, and variable numbers of *B. campestris* chromosomes (Yamashita et al. 1989)
Metaphase chromosomes were hybridized with biotin-labeled DNA probes, and the hybridization was detected by using alkaline phosphatase reaction as described in "Materials and methods"
NT: not tested

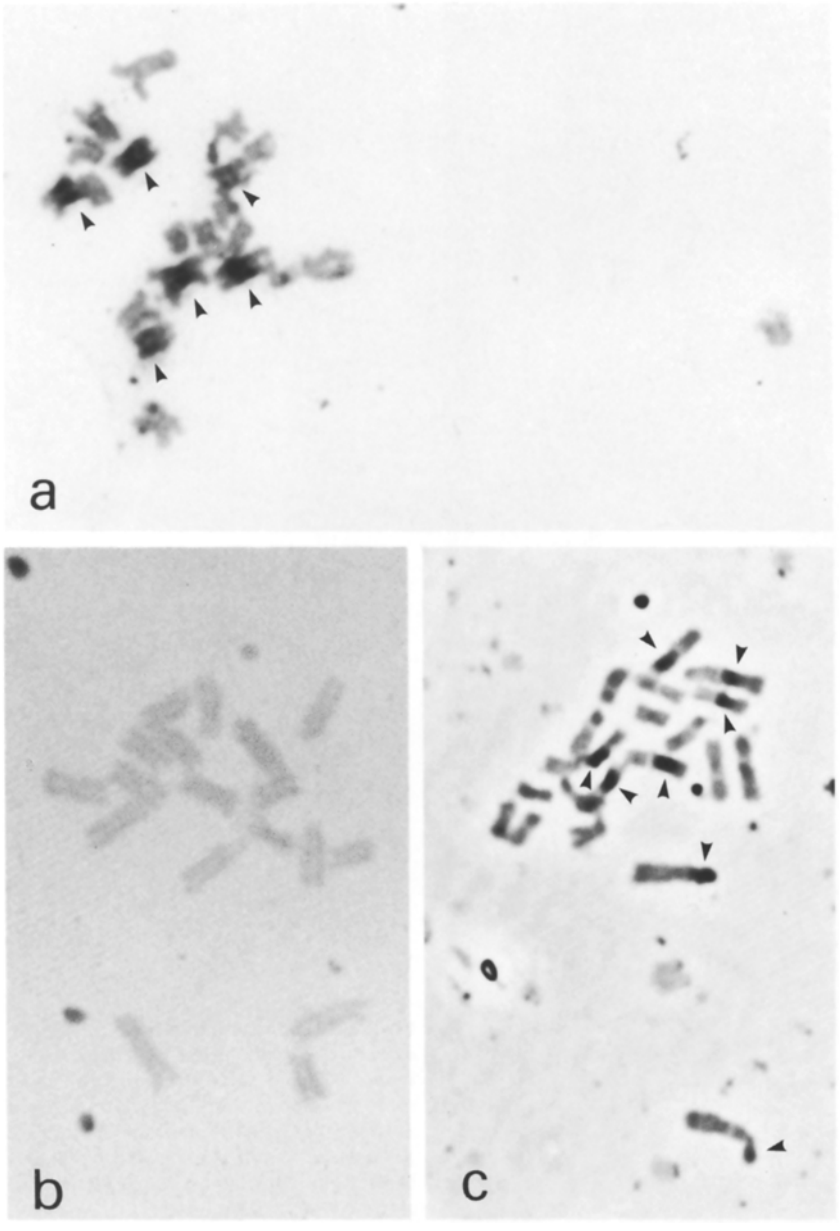

Fig. 2a–c. In situ hybridization with repetitive DNA probes shows species specificity at the chromosome level. Methods of chromosome preparations and hybridization are described in "Materials and methods". **a** *B. campestris* chromosomes (2n = 20) hybridized with CS1. **b** *B. oleracea* chromosomes (2n = 18) hybridized with a mixed probe CS1 + CT10. **c** *B. campestris* chromosomes hybridized with a mixed probe CS1 + CT10. *Arrowheads* point to the regions showing hybridization

periments confirmed the specificity of the probes used at the level of hybridization with metaphase chromosomes.

Five independent somatic hybrids obtained by fusion of *B. oleracea* protoplasts with X-irradiated *B. campestris* protoplasts were analyzed by in situ hybridization using the two different hybridization probes (Table 1; Fig. 3). They are all aneuploids, except for H7, and they possess various numbers of chromosomes. Our previous study suggests that all the hybrids except for H7 appear to be asymmetric hybrids, in which a partial genome of *B. campestris* is transferred to *B. oleracea* (Yamashita et al. 1989).

To examine whether the number of *B. campestris* chromosomes is indeed reduced in the hybrids, their metaphase chromosomes were examined by in situ hybridization. When a mixture of CS1 and CT10 was used as a DNA probe, five to seven *B. campestris* chromosomes were identified in the four hybrids, H8, H12, H13 (Fig. 3a), and H19, whereas H7 (Fig. 3b) contained 11 chromosomes hybridized with the CS1 probe. Because a complete *B. campestris* genome should contain eight chromosomes hybridized with the mixed probe, it was concluded that at least one to three chromosomes of *B. campestris* that can be identified by in situ hybridization with the mixed probe are missing in the hybrids examined. This result demonstrates the asymmetric nature of those hybrids examined. The hybrid H7, which possesses 56 chromosomes, was initially considered to be

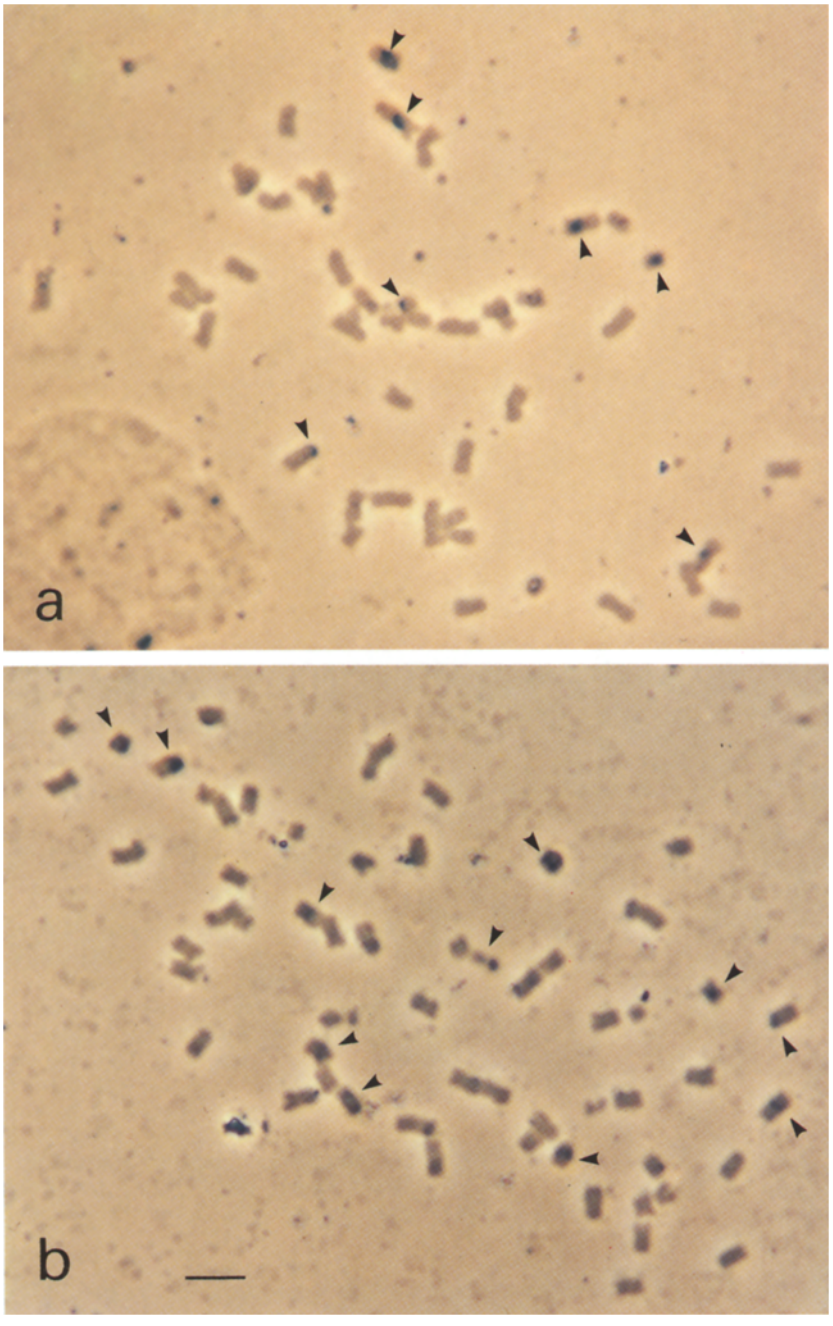

Fig. 3a and b. In situ hybridization of a *B. campestris*-specific probe with somatic hybrids, H13 and H7. Conditions for chromosome preparations and hybridization are described in "Materials and methods". *Arrowheads* point to regions with hybridization. Bar is 5 μm. **a** Metaphase chromosomes of hybrid H13. A mixed probe with CS1 + CT10 was used as a hybridization probe. **b** Metaphase chromosomes of hybrid H7. CS1 was used as a hybridization probe

a normal hybrid containing one set of *B. campestris* chromosomes (2n = 20) and two sets the of *B. oleracea* genome (2n = 18). In situ hybridization showed, however, that the number of chromosomes hybridized with CS1 was 11, while diploid *B. campestris* contains six identifiable chromosomes. This suggested that the hybrid contains almost two sets of diploid *B. campestris* chromosomes and that our initial inferrence about its genomic constitution is not correct. More likely, this hybrid resulted from fusion of one *B. oleracea* genome and two *B. campestris* genomes, from which some chromosomes were eliminated due to X-irradiation prior to cell fusion. Based on this result on in situ hybridization, we concluded that the hybrid H7 is also an asymmetric hybrid.

The results of in situ hybridization clearly showed that all five somatic hybrids examined lost some *B. campestris* chromosomes. Moreover, one hybrid that was not considered to be an asymmetric hybrid by our previous analysis turned out to be an asymmetric hybrid by the analysis of in situ hybridization.

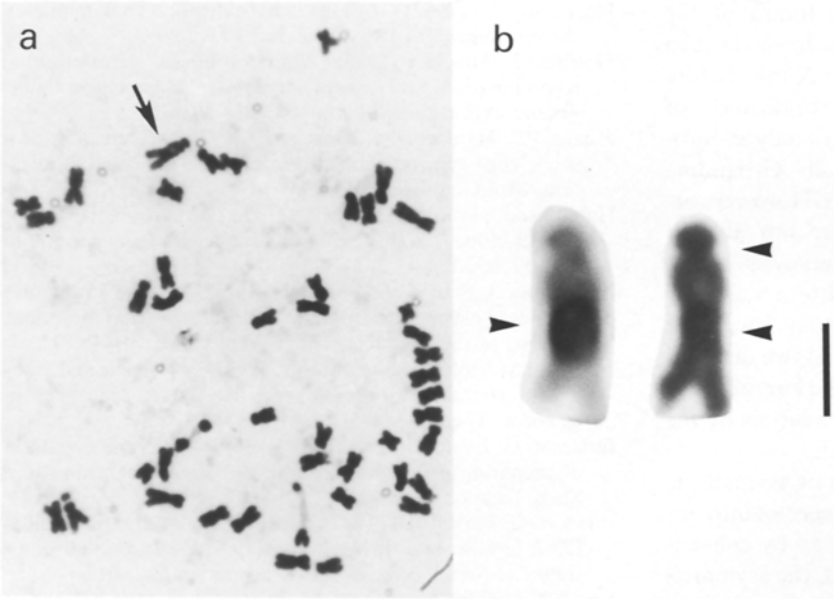

Fig. 4 a and b. Detection of an aberrant chromosome in hybrid H12 by Giemsa-staining and in situ hybridization. Methods for Giemsa-staining and in situ hybridization of chromosomes in hybrids are described in "Materials and methods". **a** Metaphase chromosomes of hybrid H12 stained by Giemsa. *Arrow* points to a dicentric chromosome. **b** A dicentric chromosome hybridized with CS1 + CS10 (left). *Arrowhead* indicates one of the two centromere regions showing a hybridization signal. At the right is a Giemsa-stained dicentric chromosome. *Arrowheads* point to the centromeres. Bar is 5 μm

Analysis of chromosomes with aberrant structures

Analysis of the hybrids by in situ hybridization revealed the presence of chromosomes with abnormal morphology that are not detected in either of the parents used for cell fusion. In the hybrid H12, one dicentric chromosome was found (Fig. 4a). In situ hybridization with a mixed probe containing CS1 and CT10 exhibited strong hybridization around one of the centromeres. This demonstrated that at least part of this aberrant chromosome was derived from *B. campestris*, presumably due to translocation of fragmented chromosomes. In the other hybrids examined, similar aberrant chromosomes exhibiting strong hybridization at the centromere region were observed (results not shown). This result demonstrated that in situ hybridization with species-specific DNA probes is also useful to identify the origin of abnormal chromosomes often found in those somatic hybrids produced between two distantly related species.

Discussion

In this investigation, we demonstrated that combination of species-specific DNA probes and in situ hybridization is a useful tool to analyze chromosomes of somatic hybrids in which changes in the karyotype are often observed. We were also able to demonstrate that this technique can identify the origin of those chromosomes with various types of structural alterations such as deletion and translocation.

One of the critical factors influencing results in in situ hybridization with smaller plant chromosomes is whether or not chromosome morphology is kept intact after chromosomal DNA is denaturated. In our study, to keep morphology of chromosomes after DNA denaturation, we dried slides slowly in a desiccator while applying a standard denaturation method with formamide. In our experiments, optimal time for denaturation of chromosomal DNA greatly changed, depending on the "age" of the chromosome preparations. For the slides kept in a desiccator for less than 2 weeks, shorter denaturation time was necessary. However, better results were generally obtained with aged slides than with those kept for shorter time. To obtain good results with freshly prepared slides, use of NaOH/ethanol as a denaturation solution appears to be more suitable than the standard formamide solution (Visser et al. 1988; Lomholt et al. 1989).

Another important step in our protocol is the use of ethidium bromide in addition to colchicine for pretreatment of root tips. The reason for inclusion of ethidium bromide in the pretreatment solution is to obtain prometaphase-early metaphase chromosomes which retain characteristics of individual chromosomes. Also they tend to keep the morphology after DNA denaturation. We were not able to obtain satisfactory results when 8-hydroxyquinolin was used for pretreatment.

Two DNA probes used in this study were not able to identify all the *B. campestris* chromosomes. Nevertheless, it was clearly shown that the aneuploidy found in all five of the hybrids examined was due to elimination of *B. campestris* chromosomes resulting from X-irradiation. Similar chromosome elimination was likely to take place in the other hybrids, which were not studied in this investigation (Yamashita et al. 1989). It is obvious that more independent clones of *B. campestris*-specific repetitive sequences need to be isolated in order to make extensive analysis of chromosomal constitutions of the hybrids.

Analysis of aberrant chromosomes found in the hybrids suggested that they originate from broken *B. campestris* chromosomes produced by X-ray. Before we established a protocol for in situ hybridization of *Brassica* chromosomes, we attempted to analyze chromosomes of the hybrids by the trypsin G-banding method (Drewry 1982; Fukui et al. 1987). However, because of the following reasons, we were not able to successfully use this technique for the analysis of the hybrids. First, characteristic banding pattern was only observed in *B. campestris* chromosomes and was not found in *B. oleracea* chromosomes. Second, we detected a number of small structural changes in the karyotype of the hybrids. This made the chromosome analysis by the banding method complicated and difficult.

It has been proposed that production of asymmetric hybrids transfers important traits of one species into another when those species cannot be crossed by conventional methods (Dudits et al. 1987). In fact, the asymmetric hybrids of *B. oleracea* and *B. campestris* examined in this study were backcrossed to *B. oleracea* twice, and fully fertile plants having all the morphological and agronomic characteristics of *B. oleracea* have been now obtained. Furthermore, these "*B. oleracea*" plants originating from the asymmetric somatic hybrids express a disease resistance that is derived from the *B. campestris* parent. It will be interesting to analyze the chromosomes of these plants by in situ hybridization, in order to examine whether parts of *B. campestris* chromosomes are indeed contained in the chromosomes of *B. oleracea*.

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