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

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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 Lonsdale 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,
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. *pekinesis* 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 SalI or TaqI was cloned at the SalI or AccI 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 × SSC (1 × SSC = 50 mM sodium phosphate, pH 6.8, 120 mM NaCl, 15 mM 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% colchicine 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 mM KCl, and 7.5 mM 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 weeks 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 (Amer sham).

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 Gil (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 mM NaCl, 100 mM 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 mM MgCl2, 100 mM NaCl, 100 mM 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 Optiphot). For photographing, Fujichrome DX100 and Minicopy HRII were used.