組み合わせ染色体処理と画像法による姉妹染色分体交換位置の同定

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Detection of sister chromatid exchange sites by sequential staining and imaging methods

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ABSTRACT

A simplified fluorescence plus giemsa method for demonstrating sister chromatid differentiation has been developed in conjunction with a C-banding method for the same barley metaphase chromosomal spread. By combining this method with imaging methods, the sites of sister chromatid exchanges could be exactly determined.

1. INTRODUCTION

Since the first detailed demonstration of sister chromatid differentiation (SCD) by the fluorescence plus giemsa (FPG) method (Perry and Wolff, 1974) in plant chromosomes was reported by Kihlman and Kronborg (1975), several studies have been conducted in order to detect the sites of sister chromatid exchange (SCE). Even though SCE sites were detected directly in human chromosomes by the sequential staining method (Latt, 1974; Fuster et al., 1992), there have been few studies on the direct detection of SCE sites in plants. The morphological features of chromosomes, such as the satellites and telomeric regions or segmentation using relative lengths (Schubert et al., 1979, 1980), have been used to define the regional differences in the SCE frequencies in plant chromosomes (Schvartzman and Cortés, 1977; Friebe, 1978).

Here, we describe a new simplified FPG method which, in conjunction with sequential C-banding to the same metaphase spread, enables the direct and exact detection of SCE sites. Using this improved method, together with image analysis methods, the SCE sites were directly localized to the defined chromosomal regions determined by the C-banding pattern.

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2. MATERIALS AND METHODS

Plant materials and sample preparation

Roots of germinated barley (*Hordeum vulgare* L. cv. Kashimamugi) were used for the study. When the roots reached 2–3 cm in length, the germinating seeds were first treated with a solution containing 100 μM 5-bromo-2′-deoxyuridine (Sigma) and 200 μM deoxycytidine (Sigma) for 12 h and, after rinsing with distilled water for 10 min, they were dipped in a solution containing 100 μM thymidine (Sigma) and 200 μM deoxycytidine for 12 h. The root tips were then excised and pretreated with a 0.01% colchicine solution for 3 h. All the treatments were carried out at 25°C in the dark. The root tips were subsequently fixed with a freshly prepared Farmer's solution (methanol : acetic acid=3:1) and were stored at −20°C for about a week. For chromosome sample preparation, the root tips were rinsed with distilled water, and were macerated with an enzymatic mixture [4% (w/v) Cellulase "Onozuka" RS, Yakult Honsha Co., Ltd., Tokyo; 2% (w/v) Macerozyme R200, Yakult; 1% (w/v) Pectlyase Y-23, Seishin Pharmaceutical Co., Ltd., Tokyo; 75 mM KCl; 7.5 mM Na_2EDTA; pH 4.0] for 13 min at 37°C. After rinsing, the root tips were chopped into fine pieces adding a new fixative, flame-dried (Fukui and Kakeda, 1990), and air dried over-night.

Fluorescence plus Giemsa staining

Chromosome samples were stained with a 0.01% fluorochrome, H33258 (Calbiochem) solution diluted with McIlvaine's buffer (6.6 mM citric acid; 88 mM Na_2HPO_4; pH 7.0) at 4°C for 15 min. After rinsing with 2×SSC (0.3 M NaCl; 0.03 M sodium citrate; pH 7.4) twice at 25°C, the samples were briefly dipped 10 times in 2×SSC at 60°C, and then mounted with 2×SSC at 60°C. Cover slips were in 0.12–0.17 mm thickness (Matsunami No. 1, Matsunami Glass Industries Ltd., Osaka). The samples were subsequently irradiated with ultraviolet light at a distance of 3–5 mm for 5–10 min at 60°C using a trans-illuminator (HP-6L, Atto Co., Tokyo, peak wavelength of 365 nm, 700 $\mu W/cm^2$). After removal of the cover slip, the samples were dipped 5 times in 2×SSC at 60°C, and then rinsed with water for 5 min at 25°C. The slides were stained with a 4% Wright solution (Wako Pure Chemical Industries Ltd., Osaka) diluted with Sørensen phosphate buffer (67 mM Na_2HPO_4; 67 mM KH_2PO_4; pH 7.0) at 4°C for 10–20 min, and rinsed with running water. Good metaphase spreads were photographed with a black and white negative film (Neopan F, ISO32, Fuji Photo Film Co., Ltd., Tokyo).

C-banding treatment

For the C-banding treatment, the photographed slides were destained through an ethanol series (100%, 70%, 50% and 30%) for 5 min each, and then washed with distilled water. The C-banding procedure followed the method reported by

Kakeda et al. (1991) with slight modifications. Slides were treated with a 1% $Ba(OH)_2$ solution at 37°C for 1 min, rinsed with distilled water after each step of the treatments, and were stained with an 8% Wright solution diluted with Sørensen buffer at 4°C for 20 min.

Image analysis of photographic chromosome images

Chromosome images were enlarged to black and white printing paper with 1000× magnification (FM3, Fuji Photo Film Co.). The same methods as described by Fukui et al. (1989) was employed to determine the SCE sites and C-band regions by using the chromosome image analyzing system, CHIAS (Fukui, 1985, 1986, 1988). The outlines are as follows; The C-banded chromosome images were frozen in the image frame memories of the CHIAS through a TV camera, and the contour lines of the C-banded chromosomes were then extracted. The SCD chromosome images were also recorded in the other image memories, adjusting the chromosomal position exactly the same to the C-banded chromosome regions, using the extracted contour lines of the C-banded chromosomes as the overlay guide-lines. The banded regions, discriminated from the C-banded chromosome images, were superimposed onto the SCD chromosome images so as to define the SCE sites on the chromosomes.

3. RESULTS AND DISCUSSION

Fig. 1 depicts the experimental procedures from sequential staining to image analysis. The metaphase spread was stained by the FPG method (Fig. 1A), and the same spread was subsequently treated by the C-banding method (Fig. 1B). The contour lines and the banded regions were extracted from the C-banded chromosomes (Fig. 1C), and the banded regions only were superimposed onto the SCD chromosomes (Fig. 1D). As shown in Fig. 1D, most SCEs occurred at the interband regions (shown by arrows), a few SCEs were found at the junction of the band and interband (shown by solid arrow heads), and one SCE occurred at the centromeric region (shown by an open triangle).

It has been difficult to combine the conventional SCD techniques with chromosome banding methods since the chromosomes were unavoidably damaged after SCD treatment. The new simplified method that omitted the conventional long SSC treatment, made the chromosome sample endurable to the subsequent C-banding treatment. Furthermore, the enzymatic maceration method for chromosome preparation was more effective than the squashing method in avoiding coverage of cytoplasmic debris over the chromosomes. In addition, irradiation with 365 nm UV light was chosen since the UV transmissivity has been estimated to be over 98% at 365 nm, whereas it is only 0.1% at 265 nm through the glass cover slip (Technical report, Matsunami Glass Industries Ltd.). These modifications enabled sequential application of the SCD and C-banding methods to

Fig. 1. Representative steps of the sequential staining and image analysis methods for barley chromosomes. (A) SCD chromosomes prepared by the modified FPG method. (B) The same chromosome spread subjected to subsequent staining by the C-banding method. (C) Discrimination of the contour lines and the banded regions from the C-banded chromosomes. (D) Superimposition of the banded regions on to the SCD chromosomes. Bar=20 μm.

the same metaphase spread. After sequential staining, however, the C-banding pattern did not completely match the pattern to those reported previously (Fukui and Kakeda, 1990; Kakeda et al., 1991), mainly due to the lack of dot-like small bands in the C-banding pattern under the current conditions.

By use of the contour lines extracted from C-banded chromosome images by imaging methods, it became possible to combine two different chromosome images and to generate a new image with the information of two images. As a result, a metaphase spread image with both the information of SCE sites and the banded regions for each chromosome was generated (Figs. 1C, D).

Almost all the SCEs have been localized in the interband regions (C-band

negative regions) and hot spots or SCE frequent regions which were found at the regions adjacent to the heterochromatic regions in the satellite chromosomes of *Secale cereale* (Friebe, 1978) were not observed. The present results are consistent with the previous reports in barley (Schubert et al., 1980).

The sequential staining and image analysis methods described here will be useful for studying the SCE mechanism and the chromosome structure in detail.

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