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Application of EMA, Fluorescent Staining and FISH of rDNA in Analysis of *Aloe vera* (L.) Burm. f. Chromosomes

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Summary

Fluorescent banding patterns and the locations of the ribosomal RNA gene (rDNA) of Indian *Aloe vera* (L.) Burm. f. chromosomes were determined. Root tips were pretreated in 2 mM 8-hydroxyquinoline at 10 to 12 °C for 3 h. The chromosome samples were prepared by the enzymatic maceration and air-drying method (EMA). The best preparation, with all chromosomes relatively extended and well spread without cytoplasm, was obtained following enzymatic condition of 0.67% Cellulase Onozuka RS, 0.5% Macerozyme R200 and 0.1% Pectolyase Y-23 for 25 to 35 min at 37 °C. No chromomycin A_3 (CMA) positive or negative bands were detected. Five 4'-6-diamidino-2-phenylindole (DAPI) positive bands, located at centromeric regions in three small and two large chromosomes, were detected with actinomycin D treatment. The 18S-5.8S-25S rDNA sites were detected in telomeric regions of the short arm of one small chromosome and the long arm of two large chromosomes by fluorescence *in situ* hybridization (FISH).

Key words: CMA, DAPI, fluorescence *in situ* hybridization, Indian *Aloe*, karyotype

Introduction

Chromosome analysis is an important parameter to elucidate the stability of the plant at the cellular level and also for genetic and biotechnological studies. Chromosomes are the first dataset that leads to an understanding of genetics of any species.

Recently, chromosome analysis has progressed remarkably by application of the enzymatic maceration method, fluorescent staining and fluorescent *in situ* hybridization (FISH). Clearly defined chromosome samples have been obtained by the enzymatic maceration method [12]. Some morphologically similar chromosomes have been identified by banding methods using the base-specific binding fluorochrome, guanine-cytosine (GC)-specific chromomycin A_3 (CMA), and adenine-thymine (AT)-specific 4'-6-diamidino-2-phenylindole (DAPI) [18]. Detection of the ribosomal RNA gene (rDNA) sites using FISH offers molecular cytogenetic information and is essential for chromosome identification and determination of phylogenic relationships among species [7].

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The species, *Aloe vera* (L.) Burm. f., is an important genus of the family Asphodelaceae. The genus *Aloe* has nearly 400 species, mostly diploid with 2n = 14 chromosomes. *Aloe vera* which is highly important for its medicinal properties [9, 10, 13] is distributed in different countries including India. Genetic variation within the species is very distinct [16] but finding these traits on chromosomes through conventional staining techniques is not possible. Combined analysis of enzymatic maceration, CMA/DAPI staining and FISH of rDNA provide useful information for identification of each chromosome and genetic relationships [5, 14, 15].

Actually, a few studies on CMA/DAPI staining and FISH of rDNA have been reported in different species and populations of *Aloe* [1, 2]. However, those studies did not use the *Aloe vera* growing in India. Thus, considering the necessity to standardize the above mentioned chromosome analyses, the present report describes the proper condition for the enzymatic maceration methods and to characterize the chromosome by the results of combination of CMA/DAPI staining and FISH of rDNA in Indian *Aloe vera*.

Materials and methods

Indian *Aloe vera* was obtained from the Agri Horticulture Society of Kolkata, India and was grown in the experimental garden of Presidency University. Healthy root tips were collected from the plants and pretreated with 2mM 8-hydroxyquinoline for 3 hours at 10 to 12 °C, fixed in methanol-acetic acid (3:1) and stored at -20 °C.

Enzymatic maceration and air-drying (EMA) were performed according to the basic protocol of Fukui [6] with some required modifications. The root tips were placed in water at 4 °C overnight. Only the tip portions of the roots were placed in a 1.5mL microtube with enzyme mixtures. Two combinations of enzymes were examined; i) 1% Cellulase Onozuka RS, 0.75% Macerozyme R 200 (Yakult, Japan), 0.15% pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd, Japan) and 1 mM EDTA, pH 4.2 at 37 °C for 35 min and ii) 0.67% Cellulase Onozuka RS, 0.5% Macerozyme R 200, 0.1% pectolyase Y-23 and 1 mM EDTA, pH 4.2 at 37 °C for 25 to 35 min. After the treatment, root tips were carefully placed in water for 20 min and after water treatment they were placed on a clean glass slide. Excess water was blotted off and finally the root tips were tapped until the tissue became invisible with a drop of fixative taken on fine forceps. After the slides had been air-dried for more than 2 h, chromosomes were stained with 2% Giemsa solution (Merck Co., Germany) in 1/30 M phosphate buffer (pH 6.8) for 10 min, rinsed with distilled water, air dried, and then mounted with xylene. After confirmation of each chromosome's position on the slide glass, the chromosomes were destained with 70 % methanol.

Chromosomes were also stained with CMA and DAPI in accordance with Kondo and Hizume [11]. The slides were soaked for 30 min in McIlvaine buffer (pH 7.0) and treated for 10 min with distamycin A (0.1 mg·mL^{-1}). Slides were rinsed in McIlvaine buffer with 5 mM $MgSO_4$ for 10 min. The slides were stained with CMA. Some combinations of CMA concentrations and treatment durations were examined: 0.1 mg·mL^{-1} for 10, 30 and 60 min and 0.05 mg·mL^{-1} for 10 min. CMA stained chromosomes were observed under a fluorescence microscope with a BV filter cassette. After observation, the slides were destained with methanol-acetic acid (3:1) for 30 min and air-dried. For DAPI staining, two staining methods were attempted. i) Slides were soaked for 30 min in McIlvaine buffer (pH 7.0) and treated for 10 min with actinomycin D (0.25 mg·mL^{-1}). The slides were rinsed in McIlvaine buffer for 5, 10 and 15 min and stained with DAPI (0.1 μg·mL^{-1}). ii) The slides were stained with DAPI (0.1 μg·mL^{-1}) for 10 and 30 min without actinomycin D treatment. DAPI stained chromosomes were observed under a fluorescence microscope with a UV filter cassette. After observation, the slides were destained

with methanol-acetic acid (3:1) for 30 min and air-dried.

For detection of the 18S-5.8S-25S rDNA sites, a 9.0 kb fragment including a full length 18S-5.8S-25S rDNA repeat unit of wheat [3, 8] was used as a probe. The rDNA probes obtained were labeled with biotin-16-dUTP (Roche, Manheim, Germany).

FISH was performed according to the method of Ohmido and Fukui [17] with slight modifications [21]. The biotinylated probe was hybridized to chromosomal rDNA *in situ* and detected with a fluorescein isothiocyanate (FITC)-avidin conjugate (Vector, California, USA) by fluorescence microscopy. FITC signals were visualized using a B filter. Chromosomes were counterstained with 0.5 $\mu g \cdot mL^{-1}$ PI and visualized using a G filter.

Results and Discussion

Adequate enzyme maceration conditions for chromosome preparations of *Aloe vera* have not been elucidated though its chromosomes were prepared by enzymatic maceration in a previous report [1]. Thus, we investigated how to develop the conditions of the enzymatic composition and duration of incubation. First, an enzyme mixture containing 1% Cellulase Onozuka RS, 0.75% Macerozyme R 200 and 0.15% pectolyase Y-23 for 35 min was attempted. In these conditions, root tips became too soft and chromosomes were sometime lost due to excessive enzyme concentration. Next, an enzyme mixture containing 0.67% Cellulase Onozuka RS, 0.5% Macerozyme R 200 and 0.1% pectolyase Y-23 for 25 to 35 min was used. The best preparation, with all 14, six small and eight large, chromosomes, relatively

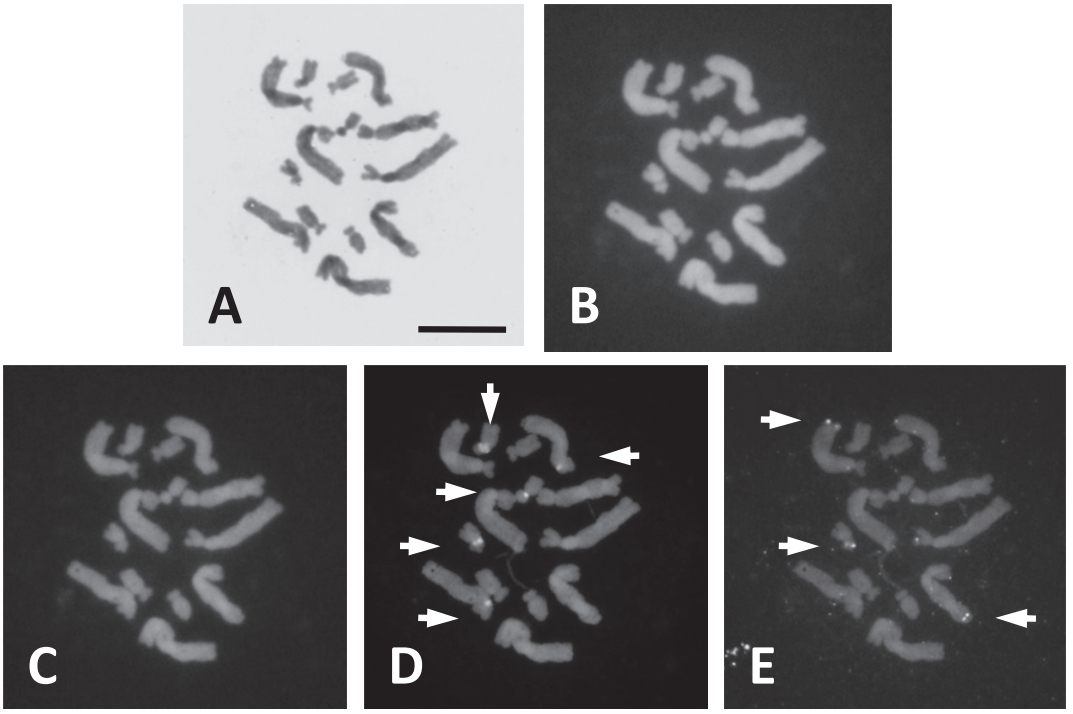

Figure 1. Somatic chromosomes of Indian *Aloe vera* prepared by the enzymatic maceration and air-drying method. A: stained with Giemsa, B: stained with CMA, C: stained with DAPI without actinomycin D, D: stained with DAPI with actinomycin D, and E: FISH of 18S-5.8S-25S rDNA. Arrow indicates DAPI positive bands and rDNA sites in D and E, respectively. Bar in A represents 20 μm for all figures.

extended and well spread without cytoplasm was observed in these conditions (Fig. 1). Therefore, all chromosome samples used in subsequent CMA, DAPI and FISH analyses were prepared under these conditions.

Chromosomes were stained with four combinations of CMA concentrations and durations (stained lightly to heavily). In any conditions, CMA positive or negative bands were not detected in the *Aloe vera* used in this study (Fig. 1B). When chromosomes were stained with DAPI without actinomycin D treatment, neither DAPI positive nor negative bands appeared (Fig. 1C). On the other hand, three and two distinct DAPI positive bands were detected at centromeric regions of small and large chromosomes respectively, when they were stained with DAPI with actinomycin D treatment (Fig. 1D). DAPI positive bands were the brightest with 15 min staining. It was demonstrated that negative counterstaining with actinomycin D sometimes increases the contrast of DAPI fluorescence in chromosomes [18]. The actinomycin treatment is essential for detecting clear DAPI positive bands in *Aloe vera* though some plants showed DAPI positive or negative band without actinomycin D treatment [5, 20].

Since CMA is GC- and DAPI is AT-specific fluorochrome, CMA positive or negative bands usually correspond DAPI negative or positive bands, respectively [11, 19, 20]. However, there is an exception [4]. In some *Aloe* species [2], not all the regions of DAPI positive bands were detected as CMA negative bands. These results agree with that of the present study on Indian *Aloe vera* as a material.

In situ hybridization with the 18S-5.8S-25S rDNA probe revealed signals on three chromosomes of *Aloe vera*. The one and two signal sites were located in telomeric regions of the short and long arm of one small and two large chromosomes, respectively (Fig. 1E). DAPI positive bands were located in one short chromosome possessing 18S-5.8S-25S rDNA site (Fig. 1E). Fig. 2 illustrates the schematic representation of DAPI positive bands and 18S-5.8S-25S rDNA sites in somatic chromosomes of Indian *Aloe vera*. This is a first combination result of DAPI banding pattern and rDNA sites. While, DAPI positive bands are located at three out of six small and two out of eight large chromosomes, 18S-5.8S-25S rDNA sites are located at one out of six small and two out of eight large chromosomes. Neither the DAPI positive band nor 18S-5.8S-25S rDNA site are detected in three and four of small and large chromosomes, respectively. Some chromosomes could be distinguished easily based on the combinations of DAPI staining and FISH of 18S-5.8S-25S rDNA.

CMA and DAPI staining on cultivars of Bangladeshi *Aloe vera* have been reported by Alam and Khaman [2]. They detected three CMA negative and seven DAPI positive bands. DAPI positive bands were located at centromeric regions of three small chromosomes in both the studies. However, the number of DAPI positive bands on large chromosomes was different; two and four in present and previous studies, respectively. A difference in the numbers of CMA negative bands was also detected. The DAPI banding pattern of the present study and that of the previous study was similar but not identical. The numbers of 18S-5.8S-25S rDNA sites of the previous study on *Aloe vera* of the Arabian peninsula [1] dif-

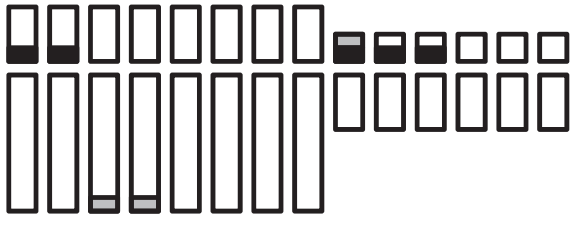

Figure 2. Schematic representation of DAPI positive bands and 18S-5.8S-25S rDNA sites in somatic chromosomes of Indian *Aloe vera*. The black and grey regions indicate DAPI positive bands and rDNA sites, respectively.

fered from that of the present study on Indian *Aloe vera* also: one at the short arm of a small chromosome and two at the long arm of large chromosomes in the present study whereas two at the short arm of small chromosomes and four at the long arm of large chromosomes in the study of Adams *et al.* [1]. Both previous studies [1, 2] reported the interspecific difference of the location of CMA and DAPI band and 18S-5.8S-25S rDNA sites in *Aloe*. Moreover, their locations were variable within *Aloe vera* from the results of the present and previous studies [1, 2].

In conclusion, we could establish a method of EMA technique and sequential analyses for fluorescent banding and FISH of rDNA in *Aloe vera*. It invites extensive work on this very important medicinal plant for karyotyping, elucidation of diversity on the chromosome configuration of this genera and species and to prepare a fluorescent chromosomal database for future use.

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Aloe vera（L.）Burm. f.の染色体分析におけるEMA法，蛍光染色及びrDNAのFISHの適用

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要　約

Aloe vera（L.）Burm. f.（アロエ）染色体の蛍光分染及び染色体上でのリボゾームRNA遺伝子（rDNA）の位置の決定を実施した。

根端は2 mMの8-hydroxyquinolineで10～12 °C，3時間前処理した。染色体標本は酵素解離空気乾燥法（EMA）によって作製した。全染色体が適度に拡がり，細胞質の無い最も良好な標本は，以下の条件で得られた；0.67%のCellulase Onozuka RS，0.5%のMacerozyme R200及び0.1% Pectolyase Y-23で25～35分，37 °C。

Chromomycin A_3（CMA）バンドは検出できなかった。Actinomycin D処理を併用すると，4’-6-diamidino-2-phenylindole（DAPI）＋バンドが小染色体の動原部に3か所，大染色体の端部に2か所の計5か所に出現した。

蛍光*in situ*ハイブリダイゼーション（FISH）によって，18S-5.8S-25S rDNAは1本の小染色体の単腕端部に1か所，2本の大染色体の長腕端部に各1か所位置することが明らかとなった。

キーワード：CMA，DAPI，蛍光*in situ*ハイブリダイゼーション，核型分析

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