A Comparative Study of the Three Cucumber Cultivars Using Fluorescent Staining and Fluorescence In Situ Hybridization

Yoshikazu Hoshi 1*, Kouhei Yagi 2, Masaki Matsuda 3, Hideyuki Matoba 4, Norikazu Tagashira 5, Wojciech Plaźder 2, Stefan Malepszy 2, Katsuya Nagano 1 and Atsumi Morikawa 6

1 Department of Plant Science, School of Agriculture, Tokai University, Kawayo, Minamiaso-mura, Aso-gun, Kumamoto 869–1404, Japan
2 Department of Plant Genetics, Breeding and Biotechnology, Faculty of Horticulture and Landscape Architecture, Warsaw Agricultural University, ul. Nowoursynowska 159, Warsaw 02–787, Poland
3 Course of Agricultural Sciences, Graduate School of Agriculture, Tokai University, Kawayo, Minamiaso-mura, Aso-gun, Kumamoto 869–1404, Japan
4 Department of Applied Biological Science, College of Bioresource Sciences, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252–8510, Japan
5 Department of Living Design and Information Science, Faculty of Human Life Science, Hiroshima Jogakuin University, 4–13–1 Ushita-Higashi, Higashi-ku, Hiroshima 732–0063, Japan
6 Agricultural Training Station, School of Agriculture, Tokai University, Kawayo, Minamiaso-mura, Aso-gun, Kumamoto 869–1404, Japan

Received October 28, 2010; accepted November 23, 2010

Summary A molecular cytogenetic survey of Cucumis sativus L. revealed a chromosomal polymorphism among 2 European pickling cultivars of ‘Borszczagowski’ and ‘Monastyrski’, and one Japanese cultivar of ‘Zibai’ by fluorescent banding method with chromomycin A 3 (CMA) and 4’,6-diamidino-2-phenylindole (DAPI), and fluorescence in situ hybridization (FISH) with the probes of 5S, 45S rDNA and telomere repeat sequences. Heterochromatic banding polymorphism was found in some chromosomes with CMA and DAPI staining, whereas no chromosomal polymorphism was found in FISH. Thus, the FISH allowed us to identify all of homologous chromosomes in each cucumber cultivars, while CMA and DAPI banding showed an easy detection of the polymorphism in cucumber chromosomes, even in close related cultivars between European pickling cultivars.

Key words CMA, DAPI, Chromosomal polymorphism, Cucumis sativus, rDNA, Telomere, FISH.

Cucumber, cucumis sativus L., is one of the most important crops, agriculturally and economically, in the world (Jeffrey 1980, Pitrat et al. 1999). A draft of the genome for the cucumber (2n=2x=14) was recently published, having been determined used next-generation DNA sequencing technologies (Huang et al. 2009). In response to the need in basic science and genomics for an understanding of the important plants, the genomic characteristics of the cucumber have been investigated using cytogenetic and molecular genetic methods. (Turkov et al. 1974, Ramachandran and Seshadri 1984, Plaźder et al. 1998, Hoshi et al. 1998, 1999, Chen et al. 1998, Koo et al. 2002, 2005, Han et al. 2008).

In spite of the importance of cytological study, cucumber chromosome studies are difficult due to the small size and poor stainability with conventional staining. In cytogenetics, the first karyomorphological studies for chromosomal identification were reported by Turkov et al. (1974), and
subsequently several attempts, including C-banding, were performed for the exact identification of the cucumber’s chromosome. The C-banding method which visualized heterochromatic regions was used for cucumber chromosomes, and this method was able to identify all of the chromosomes by their banding patterns (Ramachandran and Seshadri 1984, Hoshi et al. 1998, Chen et al. 1998, Koo et al. 2002).

In contrast to C-banding, fluorescence banding methods have been often used for chromosomal identification in cucumber due to conventional and stable high stainability (Pla˛der et al. 1998, Hoshi et al. 2008). In particular, fluorescent sequential staining of GC-specific chromomycin A₃ (CMA) and AT-specific 4’,6-diamidino-2-phenylindole (DAPI) banding have been developed as successfully reproducible methods for showing many banding characteristics on identified individual chromosomes of cucumber (Pla˛der et al. 1998, Hoshi et al. 2008). The base-specific fluorescence dyes have been employed in other crops and vegetable to investigate chromosomal polymorphism (e.g., Fukui et al. 1994, Hayasaki et al. 2001).

FISH is a powerful tool for the detection of the position of a target DNA sequence on the chromosome, and is carried out for chromosome identification in many important plants (Kato et al. 2004). Fluorescence dyes, especially DAPI, are normally used for counter staining in the FISH method (Moscone et al. 1999, Hizume et al. 2002). Although banding patterns of counterstaining in FISH are shown in some plant chromosomes, the counterstained chromosomes cannot have effective information for characterizing or identifying, due to chromosomal denaturing during the FISH process (Heng and Tsui 1993). In the case of rice, for example, since chromosomes before FISH have more informative characteristics for identification than those counterstained after FISH, the banding pattern analysis is performed with Giemsa stained chromosomes at first, and then physical mapping on the same chromosomes is applied using FISH (Shishido et al. 2000). In a plant which shows many banding characteristics on the chromosomes by conventional staining, to detect a gene or certain DNA locations has the advantage to characterize evaluation before FISH chromosomes.

In the cucumber, however, there has been no report as the performance to combine sequential fluorescent staining and FISH on the same chromosomes, even though many bands appeared on the chromosome complements by the simple fluorescence method. Therefore, it is important to develop the combined technique for gene mapping on chromosomes characterized in detail.

In the present work, in order to obtain more efficient information for detecting intraspecific chromosomal polymorphism and for physical mappings among cucumber varieties, we used 2 European pickling types and one Japanese type, and determined the locations of the 5S, 45S rDNAs and telomere repeats after banding analyzed chromosome by sequential fluorescence with CMA and DAPI.

Materials and methods

Plant materials

Two pickling-type cucumber cultivars, Cucumis sativus ‘Borszczagowski’ and C. sativus ‘Monastyrski’ and one South China-type cucumber cultivar C. sativus ‘Zibai’ were cultivated in the Department of Plant Science, School of Agriculture, Tokai University.

Chromosome preparation

Mitotic chromosome slides were prepared using the enzymatic digestion method. For fluorescent staining and FISH, slides were prepared according to a previous method (Hoshi et al. 2008). The root tips for chromosome preparation were collected from seedlings. Root tips were treated with 0.05% colchicine at 18°C for 2 h and fixed in 3:1 ethanol–acetic acid for 1 h and washed with distilled water for 1 h at 4°C. Those roots were cut into 2 mm in length and treated
with 4% Cellulase ‘Onozuka’ R10 (Yakult) and 2% Pectolyase Y-23 (Kikkoman) at pH 4.8, 37°C for 1 h. They were then washed with distilled water at 4°C for 1 h, and chopped on a glass slide with 3 : 1 ethanol–acetic acid.

**Differential fluorescent staining with CMA and DAPI**

For fluorescent differential staining with CMA and DAPI, the method of Plaδder et al. (1998) was followed with slight modifications. The air-dried slides were treated with McIlvaine’s buffer (pH 7.0) for 30 min, 0.1 mg/ml CMA in McIlvaine’s buffer supplemented with 5 mM MgSO₄ for 10 min at room temperature under dark conditions, and then mounted by glycerol for at least 10 h. The chromosome preparations were observed with BV filter cassette and fluoresced yellow. After the observation, the preparations were destained in 45% acetic acid for 30 min, and ethanol series (30%, 70% and 99.5% (v/v)) for 10 min, respectively. They were treated with McIlvaine’s buffer for 30 min. Then, the slides were stained with 0.1 μg/ml DAPI in McIlvaine’s buffer for 10 min. After the treatment, they were irradiated with U filter cassette and fluoresced blue. Photographs were taken with SenSys (Photometrics) and Olympus fluorescent microscope (BX51). Chromosome identification followed the procedure of Hoshi et al. (1999). Definitions of prometaphase, early-metaphase and mid-metaphase stages in somatic cells followed Plaδder et al. (1998), and Hoshi et al. (2008).

**PCR amplification of 5S, 18S rDNA and telomere repeat sequences**

Total genomic DNA was extracted from young leaves of *C. sativus* ‘Borszczagowski’ according to the method of Shaw (1988). PCR amplification of 5S and 18S rDNA followed the procedures of Hizume (1993) and Sogin (1990), respectively. PCR amplification of telomere sequence followed the procedure of Ijdo et al. (1991).

**FISH procedures**

The slides were pretreated with 100 μg/ml RNase in 2×SSC at 37°C for 1 to 3 h, washed in 2×SSC for 10 min. Slides were fixed in 4% paraformaldehyde at room temperature for 10 min, and denatured at 72°C for 30 s in 70% formamide at 37°C. Then, the slides were rapidly-cooled in ethanol series (70%, 99.5%) at −20°C. The PCR fragments of 5S rDNA and the telomere probe were labeled with digoxigenin-dUTP (Roche), and 18S rDNA was labeled with biotin-14-dATP (Invitorogen) by means of random primer DNA labeling. Hybridization mixtures contained 50% formamide, 10% dextran sulfate and each labeled DNA or fragment at a concentration of 4 ng/μl in 2×SSC. Twenty-five microliters of hybridization mixture was put on a chromosomal preparation and covered with a cover slip and sealed with rubber gum. The slides were denatured at 80°C for 3 min on hot plate and then incubated overnight at 37°C in humid chamber. After overnight incubation, cover slips were floated off in 2×SSC and slides were washed in 2×SSC at 42°C for 10 min, 0.2×SSC at 42°C for 10 min, and 2×SSC with 0.2% Tween20 at room temperature for 10 min twice. The slides were blocked with 5% (w/v) bovine serum albumen in 2×SSC with 0.2% Tween20 for 10 min at room temperature. Signals were detected with 100 μl of 5 μg/ml antidigoxigenin-rhodamine (Roche) and 2 μg/ml avidin-FITC (Roche) in 2×SSC in each slide for 1 h at 37°C in humid chamber. The slides were washed in 2×SSC with 0.2% Tween20 for 10 min twice, 2×SSC at room temperature. The slides were then mounted in Vectashield mounting medium (Vector Lab.) containing 0.5 μg/ml DAPI. Pseudocolor images were synthesized by overlaying the fluorescent staining images and FISH signals from each probe on the DAPI fluorescence images.
Results and discussion

The results of CMA and DAPI band patterns in 3 cultivars of cucumber are shown in Table 1 and Figs. 1–4. Prometaphase chromosomes stained with CMA and DAPI were mainly analyzed for the comparative study. Most of the chromosomes displayed CMA-positive and DAPI-negative (CMA+/H11001 DAPI-/H11002) and CMA-positive and DAPI-positive (CMA+/H11001 DAPI+/H11001) constitutive heterochromatic regions at prometaphase (Table 1, Figs. 1, 2, 4). These heterochromatic regions were mainly located on terminal and proximal regions of the chromosomes. With C. sativus

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Banding character</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Borszczagowski</td>
<td>CMA+DAPI+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CMA+DAPI−</td>
<td>2</td>
</tr>
<tr>
<td>Monastyrski</td>
<td>CMA+DAPI+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CMA+DAPI−</td>
<td>2</td>
</tr>
<tr>
<td>Zibai</td>
<td>CMA+DAPI+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CMA+DAPI−</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. Fluorescent band number of prometaphase chromosomes defined by CMA and DAPI staining in 3 cultivars

![Fluorescent staining images with CMA (A–C), DAPI (D–F) and sequentially FISH images with 45S rDNA (green signals) and 5S rDNA (red signals) (G–I) of Cucumis sativus ‘Borszczagowski’ (A, D, G), ‘Monastyrski’ (B, E, H) and ‘Zibai’ (C, F, I). A scale bar represents 5 μm.](image)

Fig. 1.
'Borszczagowski’ chromosome set as the basic cucumber karyotype, prometaphase chromosomes 1 to 7 of *C. sativus* ‘Monastyrski’ and ‘Zibai’ were compared and verified (Table 1, Figs. 2, 4). Karyotype and fluorescent banding pattern of *C. sativus* ‘Monastyrski’ were quite similar, but were slight different from those of *C. sativus* ‘Borszczagowski’. Chromosomes 3 and 7 in *C. sativus* ‘Monastyrski’ were characterized by CMA and DAPI bands on the terminal regions of both arms, whereas those of *C. sativus* ‘Borszczagowski’ displayed only the band on the terminal region of long arm. In addition, CMA and DAPI heterochromatic bands of long arms in chromosomes 2 and 5 showed different sizes between the 2 pickling cultivars.
Fig. 3. *Cucumis sativus* ‘Borszczagowski’ karyotypes of sequential fluorescent staining with CMA (A), DAPI (B) and FISH with 45S rDNA (C) and telomere sequence repeats (D). Overlay chromosome image (E) was superimposed 45S rDNA and telomere sequence signals on DAPI stained image.

Fig. 4. Cytological maps with FISH signals of 45S rDNA, 5S rDNA and telomere sequence. These maps were constructed based on the ideogram of CMA/DAPI stained heterochromatic regions of ‘Borszczagowski’ (A), ‘Monastyrski’ (B) and ‘Zibai’ (C).
In contrast, the fluorescent karyomorphological characters of a Japanese cultivar (C. sativus ‘Zibai’) were quite different from those of 2 European pickling cultivars (C. sativus ‘Borszczagowski’ and ‘Monastyrski’). Our comparative result with C. sativus ‘Zibai’ and ‘Borszczagowski’ by CMA/DAPI banding patterns showed that 4 chromosomes in C. sativus ‘Zibai’ were not found in those of C. sativus ‘Borszczagowski’, although the banding patterns of the remaining 3 chromosomes of haploid genome in C. sativus ‘Zibai’ were quite similar to those of chromosomes 1, 2 and 4 in C. sativus ‘Borszczagowski’. Thus, using the CMA/DAPI fluorescent analyses, the identities of certain chromosomes of C. sativus ‘Zibai’ could not be assigned cytologically to 7 linkage groups of the previous map created by our chromosome nomenclature or numbering-system of C. sativus ‘Borszczagowski’. In other words, it indicated that the fluorescent karyotype analysis, especially CMA banding, showed large chromosomal polymorphisms among distantly related cucumber cultivars. Therefore, cytogenetic characterization by CMA banding method will be more available to determine the chromosome identity for cultivar evaluation or the parental origin of chromosomes, which contains important genes for cucumber breeding.

The results of FISH in 3 cultivars of cucumbers are shown in Figs. 1 and 2. All chromosomes in 3 cultivars could be paired and identified using the cucumber chromosome numbering system (Hoshi et al. 1999). Different sizes of 45S rDNA signals were observed at the proximal regions of the long and short arms of chromosomes 1 and 2, a smaller strong signal was located at the proximal region of the long arm of chromosome 5, and tiny signals were detected at the proximal regions of chromosomes 3 and 7 (Figs. 2, 4). One arm of chromosome 6 had a major 5S signal (Figs. 2, 4). FISH signals of telomere probe appeared at both ends of all chromosomes, and quite weak signals appeared at proximal regions of chromosomes 1, 2 and 5 (Figs. 3, 4). Non-terminal telomere repeat sequences at the internal sites are known in some cases, and are suggested to mediate chromosome fusions and fissions (Fry and Salser 1977, Meyne et al. 1990, Richards et al. 1991). In particular, internal teromeres at the proximal region near the centromere position are considered to support large karyotype change with Robertsonian fusion from telocentric chromosomes (Holmquist and Dancis 1980, Cox et al. 1993). Thus, the internal residues of telomere repeat sequences on cucumber might be an evidence of chromosome rearrangement or karyotype repatterning with their large structural changes during genome differentiation of the species.

Our result showed that no chromosomal polymorphisms among the 3 cultivars were detected by FISH. Although FISH were not enough to detect chromosome polymorphisms such as CMA fluorescent staining, FISH signals using tandemly repeated DNA with a high copy number made it possible to assign cultivar-specific chromosomes to our cytological map (Hoshi et al. 2008). On the other hand, CMA staining was a very simple procedure and revealed cultivar-specific chromosome types with multiple, fairly stable bands on a longer exposure time of the epifluorescence microscope. Therefore, cucumber, which shows many chromosome banding characteristics by conventional staining, has the advantage to characterize evaluation by fluorescent staining before FISH chromosomes. It will be useful for gene mapping on chromosomes characterized in detail, and for chromosome map integration of other important cucumber cultivars. Further cytogenetic studies by fluorescent banding and FISH analyses will be necessary to clarify a whole picture of cucumber chromosome polymorphism.

References
Fry, K. and Salser, W. 1977. Nucleotide sequences of HS-α satellite DNA from kangaroo rat dipodomys ordii and character-