Chromosomal Polymorphism of Two Pickling Cucumbers (*Cucumis sativus* L.) Revealed by Fluorescent Staining with CMA and DAPI

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Summary To resolve the discrepancy among previous karyotype descriptions, chromosome comparative study between two closely related cucumber cultivars, *C. sativus* L. cv. Borszczagowski and cv. Monastyrski, was performed by chromosome preparation technique with enzymatic maceration, and fluorescent banding method with chromomycin A₃ (CMA) and 4',6-diamidino-2-phenylindole (DAPI). Heterochromatic band polymorphisms were detected in chromosomes 2, 3, 5 and 7, whereas no chromosomal polymorphism was found in chromosomes 1, 4 and 6. In cv. Borszczagowski chromosome 2, CMA⁺DAPI⁺ heterochromatic band at terminal region of the long arm was smaller than that of cv. Monastyrski. Chromosome 3 of cv. Monastyrski was characterized by CMA⁺DAPI⁺ bands on the terminal regions of both arms, whereas that of cv. Borszczagowski displayed only on the terminal region of long arm. Distinct CMA⁺DAPI⁺ band located at terminal region of the long arm in cv. Borszczagowski was larger than that of cv. Monastyrski. In chromosome 7, terminal CMA⁺DAPI⁺ band at the short arm was only observed in cv. Monastyrski. This result suggested that inconsistency of karyotypes among previous reports was due to chromosomal polymorphism.

Key words *Cucumis sativus* L., Chromosomal polymorphism, CMA, DAPI

Cucumis sativus L. (Cucumber, $2n=2x=14$) is one of the most important crops and is widely cultivated all over the world (Jeffrey 1980). Since the first C-banding on the somatic chromosome was reported by Ramachandran and Seshadri (1986), karyomorphological studies for chromosomal identification have been going on with this species. Recently, several cytogenetists have attempted to characterize the *C. sativus* chromosomes using basic staining methods (Hoshi *et al.* 1998, Chen *et al.* 1998, Koo *et al.* 2002) and differential staining methods (Ramachandran and Seshadri 1986, Plader *et al.* 1998, Hoshi *et al.* 1998, 1999, Chen *et al.* 1998, Koo *et al.* 2002, 2005).

Ramachandran and Seshadri (1986) reported chromosome identification at mitotic metaphase and meiotic pachytene stage to compare *C. sativus* with *C. melo*. In contrast, Hoshi *et al.* (1998) and Plader *et al.* (1998) carried out the chromosome identification with new banding pattern using the improved banding method (Hoshi *et al.* 1998) and fluorescent differential staining with base-specific fluorescent dyes (Plader *et al.* 1998). Using these banding patterns, they rearranged chromosomes 1 to 7. On the other hand, Chen *et al.* (1998) applied C-banding technique for chromosome identification using a Chinese cultivar, and showed the karyotype which was different from

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those of previous reports. The latest report of the C-banding pattern was done by Koo *et al.* (2002, 2005), who used a Korean cucumber cultivar and displayed quite different pattern in the haploid karyotype ideogram. Although the differences of these karyotype information were considered due to differences of cultivar materials, chromosome preparation techniques, or growing conditions (Hoshi *et al.* 1998, Koo *et al.* 2002), an evidentially experimental investigation to advance the consideration has not been performed. In addition to this, employing the same chromosome preparation technique and banding method among different *C. sativus* cultivars are quite necessary to resolve the discrepancy among the karyotype descriptions, even though each previous banding information was enabled to characterize the own karyotype components.

The intraspecific polymorphism of chromosome or chromosome segment among different cultivars has been demonstrated in several plants such as *Secale cereale* (Weimarck 1975), *Hordeum vulgare* (Vosa 1976, Laursen 1978, Kakeda *et al.* 1991), *Scilla sibirica* (Vosa 1973a), *Leopoldia comosa* (Bentzer and Landström 1975), *Tulbaghia leucantha* (Vosa 1973b) and so on. These plants showed polymorphisms of constitutive heterochromatin revealed by C-, N- or fluorescent banding method. Moreover, base-specific fluorescent staining methods with chromomycin A₃ (CMA) and 4',6-diamidino-2-phenylindole (DAPI) have been commonly employed to localize some GC- and AT-rich heterochromatic regions on chromosome, respectively (Schweizer 1976). Plader *et al.* (1998) reported that all of *C. sativus* chromosomes were possible to easily identify based on those heterochromatic segments by the improved method for a simplified fluorescent staining. They demonstrated that the fluorescent differential staining with these base-specific fluorochromes allowed stable detection of the heterochromatic bands on chromosomes and rapid identification of each homologous chromosome pair in *C. sativus*.

In this study, we carried out comparative study of chromosomes between two European pickling type cucumber cultivars by means of fluorescent differential staining, and discussed chromosomal polymorphism.

Materials and methods

Plant material

Two pickling type cucumber cultivars, *Cucumis sativus* L. cv. Borszczagowski and *C. sativus* cv. Monastyrski were used in this study. These lines were maintained at the Department of Plant Genetics, Breeding and Biotechnology, Warsaw Agricultural University and Applied Plant Science, Tokai University.

Chromosome preparation

Mitotic chromosome slides were prepared using the enzymatic digestion method. Root tips for the chromosome preparation were collected from two day-old seedlings. The 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 Honsha) and 2% Pectolyase Y-23 (Kikkoman) at pH 4.8, 37°C for 1 h. Then, they were 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 Plader *et al.* (1998) was followed with slight modifications. The slides were pre-incubated in McIlvine's buffer (pH7.0) for 30 min, and then treated with 0.1 mg/ml CMA in McIlvine's buffer supplemented with 5 mM MgSO₄ for 10 min. After rinsing, they were mounted in glycerol and stored at 4°C for at least 10 h to prevent fading. These chromosomes stained with CMA were irradiated with a BV filter cassette

Fig. 1. Mitotic pro- (A, B) and early- (C, D) metaphase chromosome images of *C. sativus* cv. Borszczagowski stained with CMA (A, C) and DAPI (B, D). Identified chromosomes were indicated. Scale bar represents 5 μ m for A to D.

and fluoresced yellow. After CMA staining and observation, the slides were used for sequential DAPI staining. The slides were destained in 45% acetic acid for 30 min, 3 : 1 ethanol-acetic acid for 30 min and ethanol series (30%, 70% and 99.5% (v/v)) for 10 min, respectively. They were dipped in McIlvine's buffer for 30 min. And then, they were treated with 0.1 μ g/ml DAPI in McIlvine's buffer for 10 min. These chromosomes stained with DAPI were irradiated with a U filter cassette and fluoresced blue. Photographs were taken with SenSys (Photometrics) and Olympus fluorescent microscope (BX51). Chromosomes identification followed the procedure of Hoshi *et al.* (1999). Definition of prometaphase, early-metaphase and mid-metaphase stages in somatic cells followed Plader *et al.* (1998). Prometaphase chromosomes present clear banding pattern in CMA and DAPI staining. DAPI stained chromosomes do not show any clear band from early- to mid-metaphase, while the chromosomes stained with CMA show many bands.

Fig. 2. Mitotic pro- (A, B) and early- (C, D) metaphase chromosome images of *C. sativus* cv. Monastyrski stained with CMA (A, C) and DAPI (B, D). Identified chromosomes were indicated. Scale bar represents 5 μ m for A to D.

Result

Prometaphase and early-metaphase chromosomes were mainly analyzed for the comparative study. After the CMA and DAPI staining, most of the chromosomes displayed CMA-positive and DAPI-negative ($\text{CMA}^+\text{DAPI}^-$), CMA-positive and DAPI-positive ($\text{CMA}^+\text{DAPI}^+$), or CMA-negative and DAPI-positive ($\text{CMA}^-\text{DAPI}^+$) constitutive heterochromatic regions at pro-metaphase and early metaphase (Figs. 1, 2). These heterochromatic regions were mainly located on its terminal, interstitial and proximal regions of the chromosomes. With *C. sativus* cv. Borszczagowski chromosome complements as the basic *C. sativus* karyotype, prometaphase and early-metaphase chromosomes 1 to 7 of *C. sativus* cv. Monastyrski were compared and verified (Figs. 3, 4).

Prometaphase chromosome 1 of *C. sativus* cv. Borszczagowski had the largest DAPI^+ segment from the proximal to middle part of the interstitial region of long arm, and $\text{CMA}^+\text{DAPI}^-$ bands at the pericentromeric regions of both arms. The same banding pattern was observed in that of *C.*

sativus cv. Monastyrski. In contrast, early-metaphase chromosome 1 did not possess clear CMA⁺DAPI⁺ large segment. Well-defined elongated site at centromeric position of the chromosome 1 was often observed at prometaphase and early-metaphase cells of *C. sativus* cv. Borszczagowski and cv. Monastyrski.

Clear CMA⁺DAPI⁻ segments were presented at the pericentromeric regions of chromosome 2 in *C. sativus* cv. Borszczagowski and cv. Monastyrski. In *C. sativus* cv. Borszczagowski chromosome 2, the banding patterns of long and short arms were similar. In contrast to *C. sativus* cv. Borszczagowski, long and short arms of chromosome 2 of *C. sativus* cv. Monastyrski could be easily distinguished, because large CMA⁺DAPI⁺ heterochromatic band was observed at the terminal region of long arm, but not short arm.

Chromosome 3 had weakly CMA⁺DAPI⁻ band at the pericentromeric region and CMA⁺DAPI⁺ bands at the pericentromeric areas of both arms. Chromosome 3 in *C. sativus* cv. Monastyrski was characterized by CMA⁺DAPI⁺ bands on the terminal regions of both arms, whereas that of *C. sativus* cv. Borszczagowski displayed only CMA⁺DAPI⁺ band on the terminal region of long arm.

In chromosome 4, no difference was found in the banding patterns in both cultivars. The terminal region of short arm did not possess any heterochromatic bands, while the terminal region of long arm possessed large CMA⁺DAPI⁺ heterochromatic band.

Chromosome 5 showed intraspecific polymorphism between two cultivars. Distinct CMA⁺DAPI⁺ band located at terminal region of the long arm in *C. sativus* cv. Borszczagowski was larger than that of *C. sativus* cv. Monastyrski.

No heterochromatic band polymorphism was detected between chromosome 6. Chromosome 6 had CMA⁺DAPI⁺ bands at the pericentromeric and the terminal regions of short and long arms in both cultivars.

Chromosome 7, which was the smallest size in the chromosome complement, was characterized by evident CMA⁺DAPI⁺ bands at the terminal regions of long arm, and CMA⁻DAPI⁺ band located on the pericentromeric region of short arm in both of cultivars. Chromosomal polymorphism was recognized by terminal CMA⁺DAPI⁺ band, which was only observed at short arm in *C. sativus* cv. Monastyrski.

Discussion

The earliest attempt of *C. sativus* chromosome identification was reported by Ramachandran and Seshadri (1986). They reported that karyotype formula was composed of two median, four submedian and one subterminal chromosomes when Japanese type cultivar was used as plant material. More detailed karyotype analyses for chromosome identification were performed with C-banding techniques (Hoshi *et al.* 1998, Chen *et al.* 1998, Koo *et al.* 2002, 2005). Hoshi *et al.* (1998, 1999) and Plader *et al.* (1998) reported that the karyotype of European pickling type cultivar was composed of five metacentric and two submetacentric chromosomes by means of C-banding and fluorescent staining. In contrast, Chen *et al.* (1998) and Koo *et al.* (2002, 2005) reported that the haploid complement composed six metacentric and one submetacentric chromosome using North Chinese type and Korean type cultivars, respectively.

The karyotype of *C. sativus* cv. Borszczagowski in this study agreed with those of previous reports (Hoshi *et al.* 1998, 1999, Plader *et al.* 1998). However, our data was quite different from the karyotype reported by Chen *et al.* (1998) and Koo *et al.* (2002, 2005). Heterochromatin banding patterns of *C. sativus* cv. Borszczagowski chromosomes 1 and 4 were quite similar to those of chromosomes 4 and 6 in the report of Chen *et al.* (1998), and chromosomes 2 and 6 in the report of Koo *et al.* (2002, 2005), respectively. In contrast, the banding patterns of chromosomes 2, 3, 5 and 7 in the karyotype of *C. sativus* cv. Borszczagowski were not found in those of reported by Chen *et al.*

Fig. 3. Mitotic pro-metaphase karyotypes of *C. sativus* cv. Borszczagowski (A, B) and *C. sativus* cv. Monastyrski (C, D). Chromosomes were stained with CMA (A, C) and DAPI (B, D). Arrow heads were indicated chromosomal polymorphism sites between two cultivars.

Fig. 4. Mitotic early-metaphase karyotypes of *C. sativus* cv. Borszczagowski (A, B) and *C. sativus* cv. Monastyrski (C, D). Chromosomes were stained with CMA (A, C) and DAPI (B, D). Arrow heads were indicated chromosomal polymorphism sites between two cultivars.

(1998) and Koo *et al.* (2002, 2005). Our comparative result with two cultivars by using of the method for the same chromosome preparation showed that the cause of karyotype differences among cultivars is mainly due to intraspecific polymorphism.

Moreover, our study suggested that chromosome polymorphism in *C. sativus* was due to heterochromatin region structure. It is known that heterochromatic region-specific DNA is composed of highly repetitive sequences, such as *Oryza sativa* (Cheng *et al.* 2001) and *Brassica rapa* (Lim *et al.* 2005). These repetitive sequences are frequently deleted or duplicated, especially an event of unequal crossing-over during meiotic division (Smith 1976). Therefore, heterochromatic region polymorphisms in *C. sativus* might be related to this phenomenon.

The cultivated *C. sativus* is believed to be native to Western India (Candolle 1954), and includes pickling, South Chinese, North Chinese, Nepalese, and English types. There are following characteristic flute-shape in cultivation areas. Mature *C. sativus* flutes of pickling cultivars are rugby balled-shape with brown surface, while the flutes of South and North Chinese cultivars, which are most popular type in Japan, are elongated shape with green surface. Even though quite large amounts of cultivars with different morphological characters are existed in the world, it is difficult to detect genetic variation among distantly related cultivars by linkage analysis of crossing experiments (Knerr *et al.* 1989). In this study, chromosome polymorphism was detected even in closely related cultivars of pickling cucumber. Thus, we expect that karyotypes among distantly related cultivars such as Japanese type may be more variable, and then chromosome identification will be more available to detect strain variations in *C. sativus* cultivar cultivated world wide. Further comparative chromosome studies between European and Asian cultivars will be necessary to clarify a whole picture of cucumber chromosome polymorphism.

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