SHORT PAPER

Condensation pattern as a new image parameter for identification of small chromosomes in plants

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ABSTRACT

The method of using conventional numerical parameters, such as length and arm ratio, is insufficient to distinguish the chromosomes of *Atriplex rosea* L. ($2n=18$) completely, as they are very small. Therefore a new image parameter, condensation pattern (CP), was defined and employed to obtain additional information for chromosome identification. The CP of a chromosome was represented by a profile line of the density distribution along the midrib of unilateral chromatid, and was measured semi-automatically by the CHromosome Image Analyzing System, CHIAS. It was shown that the CP is a valuable parameter for identification of the small chromosomes in plants.

1. INTRODUCTION

Small chromosomes in plants are very difficult to identify by ordinary karyotype analysis, because of the following three reasons. Firstly, besides the smallness of chromosome size, chromosomal shapes are sometimes similar to each other, even in the species having large chromosomes, making karyotype analysis difficult. Secondly, stainability of chromosomes is not always good. Only N- and C-banding methods are presently available in many cases. These techniques, however, can not always be applied, especially to the species with small chromosomes, where only limited success has been obtained (Gostev and Asker, 1979; Ladizinsky *et al.*, 1979). The third difficulty is specific to plants with cells having the thick cell wall, which prevents a good spread of chromosomes by a squashing method. Moreover, tissues of *Oryza* species are siliconized, and many wood plants are ligninized making them very rigid. Therefore, the ordinary karyotype analysis has provided only limited success from the viewpoint of chromosome

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identification, not only in the plants with small chromosomes, but in many other plant species as well.

We adopted several new methods to solve these difficulties which existed also in *Atriplex rosea* L. ($2n=18$) whose average chromosomal length is around $2\text{ }\mu\text{m}$ at mitotic metaphase. We chose prometaphase chromosomes for the analysis in this study, as opposed to mid-metaphase ones which are commonly used for karyotyping. Enzymatic maceration and air drying were adopted to prepare chromosomal spreads instead of the ordinary squashing method. Image analysis of the chromosomes by the chromosome image analyzing system (CHIAS: Fukui, 1985; 1986a) was employed in order to obtain data, which are quantitatively accurate and useful for pattern recognition. Combining all these methodologies, we have been able to demonstrate a new identifying method for the plant chromosomes.

2. MATERIALS AND METHODS

Plant material

Atriplex rosea L. ($2n=18$) was kindly provided by Prof. K. Hinata, Tohoku University, Sendai 980, Japan. Seeds were sown on wet filter paper in petri dishes. Root tips were excised from germinated seeds after a five or six days incubation at 25°C under continuous illumination, and were immersed in distilled water at 0°C for 24 hours.

Cytology

Fixed root tips were macerated at 37°C for 20 minutes by using an enzymatic mixture, which contained 4% Cellulase Onozuka RS (Yakuruto Honsha Co., Ltd., Tokyo), 1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo) and 75 mM KCl and 7.5 mM Na_2EDTA , and pH was adjusted to 4.2 (Nishibayashi 1985). After the mixture was removed, few drops of Farmer's fixative were put onto slides, and the root tips were chopped into small pieces with fine needles. The slides were air dried and stained with a 3% Giemsa solution (pH 6.8) for 40 minutes. The ordinary squash method and carmine staining were also adopted in some cases. For N- and C-banding methods, usual protocols (*e.g.*, Endo and Gill, 1984) were followed, and several conditions were tested in order to obtain quality banded images.

Photographic procedures and the image analysis

Photographs were taken using the Minicopy film (ISO 6, Fuji Photo Film Co., Ltd., Tokyo). The prometaphase spread was photographed under the two different shooting conditions of ISO 6 and 10. Enlargement was also carried out under the two different conditions to obtain an overexposed print and an underexposed print each of the same prometaphase plate, by varying both exposure time and the iris set. For the metaphase spread, ordinary photographic procedures, provided by the supplier, were followed to obtain properly exposed photographs.

Image analysis was carried out using the chromosome image analyzing system, CHIAS (Fukui, 1985; 1986a). A software, to analyze the chromosomes of *A. rosea* semi-automatically, was constructed using the CHIAS.

3. RESULTS AND DISCUSSION

Fig. 1 shows a metaphase plate of *A. rosea* with eighteen chromosomes. As shown in the photograph, most of them were metacentric and almost no difference was apparently observed in the total length. The chromosomes revealed that the identification of the homologous chromosome pairs, by using the ordinary parameters of arm ratio and total length, was very difficult. Other morphological informations of the secondary constriction made it possible to assign putative four satellite chromosomes. It was, however, still difficult to pair the homologous chromosomes within four satellite chromosomes, and within the rest of fourteen chromosomes. Although a N-banding technique applied gave better results than C-banding one so far, it was not possible to attain the complete identification of them, because eight chromosomes showed a simple centromeric N-band only. All the conventional methodologies employed for chromosome identification proved insufficient in the case of *A. rosea* chromosomes.

Fig. 1. Mitotic metaphase chromosomes of *Atriplex rosea* L. ($2n=18$). The bar in the photograph indicates 2 μm .

Fig. 2. Mitotic prometaphase chromosomes of *A. rosea* L. A. An overexposed photograph for detection of dispersed telomeric regions. B. An underexposed photograph for detection of fine density distribution of each chromosome. The bar indicates 5 μ m.

Fig. 2 shows images of the same mitotic prometaphase plate produced under the two different exposure conditions, that is, overexposure and underexposure. As a result of the enzymatic maceration and the air drying methods, all chromosomes were arranged evenly on the surface of the glass slide, and were all in focus. Moreover, this method dispersed cytoplasm well, and allowed good observation of the loose telomeric regions found in the most chromosomes by the overexposure print (Fig. 2A). These uncondensed regions have been very difficult to analyze, because there has been no proper method to handle them quantitatively and reproducibly. Comparison between the underexposed photograph (Fig. 2B) and the overexposed one, however, clearly revealed the characteristic uncondensed

Fig. 3. Sequential steps of image manipulation by using the CHIAS. A. Binarization of the chromosomal regions and elimination of the dust by a man-machine interactive system. B. Discriminated chromosomes and their contour lines with a clean background. C. Super-imposition of the contour lines of the chromosomes onto the underexposed chromosomal images. D. Pseudo-color display of the enlarged chromosomes in order to clarify the primary constriction and drawing a line therein. The gray values under the polygonal lines drawn on each chromatid were measured. E. A density profiles of No. 17 and 18 chromosomes. A single profile line of the two lines based on each chromatid is shown as the representative of the chromosome. The vertical axis shows density values. There are 256 steps of the density value, where 0 is the darkest and 225 is the lightest. The horizontal axis shows number of pixels that construct the polygonal line. The numbers below the graphs are the chromosome number. Two vertical lines in the middle part of the profile line show a centromere of the chromosome. F. Idiogramatic display of condensation pattern by computer graphics of the CHIAS.

telomeric extension. Overexposure, however, obscured most of the fine structure of chromosomes including the primary constrictions of some chromosomes. Therefore, we used the overexposed photograph as the "discriminator" of the chromosomal regions, especially for the telomeric uncondensed regions. Whereas the underexposed photograph was essential as the "detector" of the fine characteristics and the density distribution of the chromosomes by image analysis.

Fig. 3 shows the flow of the images processed semi-automatically by the CHIAS. Image manipulation consisted of the following steps. Firstly, the overexposed photographic picture (Fig. 2A) was put into an image frame memory using a TV camera after analog/digital conversion. An image frame consists of 512×512 pixels, and each pixel has 8 bits (256 steps) of gray levels. Each image was averaged 20 times in order to eliminate the input noise, and to produce an original digital image for the analysis. Discrimination of the chromosomes from Fig. 2A was carried out by setting the upper and the lower thresholds of the gray level, and by establishing pixels with gray levels between the thresholds as chromosomal areas. Binarization of the gray image proceeded converting the chromosomal regions as white, and the background as black, at the same time. A digital (low-pass) filter treatment was carried out before discrimination of the chromosomes to eliminate distortions caused by a TV-camera, a zoom lens, and irregularities of illumination. Large dusts which had the gray levels between the thresholds, and discriminated as the white, were then eliminated by drawing the closed overlay lines interactively onto the binary image using a digitizer and a cursor (Fig. 3A).

Unnecessary parts were pointed by the cursor, and the dust particles were eliminated automatically. After the green overlay lines were also eliminated, the contour lines of each binary chromosome image were generated with the overlay lines to determine each chromosomal region (Fig. 3B).

Only the contour overlay lines were then transferred onto the underexposed photographic image, which had been stored in another image frame memory by the same method as the overexposed one (Fig. 3C), and overlay contour lines were copied onto the gray image as the black lines (gray level 0). Several look-up-tables, which color images differently according to the gray level value of each pixel, were tested to facilitate detection of centromeres. Look-up-table No. 1 which divided 256 steps of the gray level into 15 steps, and colored each new step differently, was selected and the image was magnified two-fold as shown in Fig. 3D. Straight overlay lines, which divided each chromosome into two arms, were drawn automatically by a cursor pointing at the both sides of the most constricted region of a chromosome. These overlay centromeric lines were also copied onto the image as the lines with the gray level 0. Thus the three different informations on the chromosome boundary, the centromeric partition, and the color-graded condensation pattern of the chromosomes were gathered into a single pseudo-colored image (Fig. 3D).

For each double size chromosome, a polygonal line along the midrib of each chromatid was drawn with an overlay line. The density of the pixels under the line was automatically picked up, and the density profile line, *i.e.*, the condensation pattern (CP), was printed out (Fig. 3E). A CP consisted of two vertical lines at both ends, which reflected the contour line with the gray level 0. Two lines in close vicinity to each other at the middle part showed the centromeric partition of the gray level 0. Chromosome pairs were decided by comparing all the 36 CPs obtained. Although we took the data of arm ratio and total length of each chromosome into account in order to pair the homologous chromosome (data not shown), we attached more importance to the CP by the following two reasons. Firstly, availability of numerical parameters at prometaphase chromosome has not been established and variability among them are often observed. Secondly, it has been preliminarily proven that each chromosome has its own condensation pattern in the case of rice chromosomes (Fukui and Iijima, 1987). Fig. 3F shows the idiogrammatic representation of the CP generated by computer graphics of the CHIAS. In the case of Fig. 3F, we defined the gray levels from 0 to 86 as the most condensed region that was shown by the color black. Dark yellow represented gray levels from 87 to 172 which corresponded to moderately condensed regions. Light yellow represented the gray levels from 173 to 255 which corresponded to the least condensed parts of the chromosomes.

Cytologists have utilized not only numerical data of arm ratio and total length, but also have observed morphological characteristics of the chromosomes, such as secondary and tertiary constrictions, distribution of condensed regions along the chromosomes, *etc.* In fact, these additional informations sometimes play an invaluable role in identifying the homologous chromosomes. Many researchers have noticed differential condensation especially amongst the chromosomes at mitotic prometaphase as a phenomenon, and some of them attempted to utilize it for chromosome identification in *Apium graveolens* (Murata and Orton, 1984), *Nicotiana glauca* (Merritt and Burns, 1974), and *Oryza sativa* (Kurata *et al.*, 1981; Fukui, 1986b). We defined condensation pattern (CP) as a new image parameter, and introduced it for identification of the plant chromosomes by using the CHIAS. It can successfully detect the minute density differences along the midrib of the ordinary stained chromatid, and the data output (CP) is obtained within one or two minutes.

By this method all of the chromosome pairs of *A. rosea* were assigned. Although it should be confirmed whether the paired chromosomes are indeed homologues or not, they are most likely homologous chromosomes, because idiogrammatic representations of the CP are also very similar to each other, as typically shown in the pair of No. 17 and No. 18 chromosomes. It was clearly shown that the CP is a valuable parameter for the chromosome study, because it could convert complicated morphological data, used to be detected by personal skill, into the numerical data that everyone can utilize. Therefore it would be

worthwhile to carry out further studies in order to establish the validity of the CP.

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REFERENCES

- ENDO, T. R. and GILL, B. S. (1984) Somatic karyotype, heterochromatin distribution and nature of chromosome differentiation in common wheat, *Triticum aestivum* L. *Chromosoma* **89**, 361-369.
- FUKUI, K. (1985) Identification of plant chromosomes by image analysis method. *The Cell* (Tokyo) **17**, 145-149.
- FUKUI, K. (1986a) Standardization of karyotyping plant chromosomes by a newly developed chromosome image analyzing system (CHIAS). *Theor. Appl. Genet.* **72**, 27-32.
- FUKUI, K. (1986b) Comparison of the Giemsa and orcein staining methods in rice chromosomes. *La Kromosomo* **II-43-44**, 1398-1404.
- FUKUI, K. and IJIMA, K. (1987) Analysis and utility of chromosome information 18. Identification of rice chromosomes by the condensation pattern (CP). *Jpn. J. Genet.* **62**, 515.
- GOSTEV, A. ASKER, S. (1979) A C-banding technique for small plant chromosomes. *Hereditas* **91**, 140-143.
- KURATA, N., IWATA, N. and OMURA, T. (1981) Karyotype analysis in rice. II. Identification of extra chromosomes in trisomic plants and banding structure on some chromosomes. *Jpn. J. Genet.* **56**, 41-50.
- LADIZINSKY, G., NEWELL, C. A. and HYMOWITZ, T. (1979) Giemsa staining of soybean chromosomes. *J. Hered.* **70**, 415-416.
- MERRITT, J. F. and BURNS, J. A. (1974) Chromosome banding in *Nicotiana glauca* without denaturation and renaturation. *J. Hered.* **65**, 101-103, 251.
- MURATA, M. and ORTON, T. J. (1984) G-band-like differentiation in mitotic prometaphase chromosomes of celery. *J. Hered.* **75**, 225-228.
- NISHIBAYASHI, S. (1985) Chromosomal analysis in rice. Conditions to observe chromosomes. *Jpn. J. Breed.* **35** (Suppl. 1), 318-319.