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Somatic chromosome map of rice by imaging methods

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Summary. Rice somatic chromosomes were completely identified and quantitatively mapped based on an image parameter, condensation pattern (CP), or a chromosomal density profile determined by imaging methods. The CP corresponds to the compactness of the chromatin fibers along the chromatid, which is characteristic in small plant chromosomes such as rice chromosomes at the mitotic pro-metaphase stage. The standard CP for every chromosome was obtained by averaging 60 CPs from 30 chromosome spreads. Each standard CP exhibited a characteristic pattern of the chromosome, which enabled it to be distinguished from the other chromosomes. An ideogram based on the numerical data and the standard CP was established. The chromosomal address was also determined based on the degree of condensation, and the fractional length of each chromosomal address was quantitatively presented.

Key words: Rice – Chromosome map – Ideogram – Image analysis – Condensation pattern

Introduction

Rice is one of the three major cereals in the world. Genetic and cytological studies have been extensively carried out for more than 100 years. Although the number of rice chromosomes ($2n=24$) was determined more than 80 years ago (Kuwada 1910), it has nonetheless been very difficult to identify every rice somatic chromosome objectively, mainly due to the small size and similarity of the karyotype of the somatic metaphase chromosomes, as well as the difficulty in making cytological preparations. Rice chromosomes are comparatively pale when they are stained with ordinary acetocarmine or acetoorcein solutions, and the tissues are also very stiff compared with many other dicots, due to the accumulation of silicates. In addition, no reproducible banding method has been available for the identification of the rice chromosomes.

In spite of these difficulties, many cytologists have attempted to identify all the somatic chromosomes of rice (Nandi 1936; Hu 1958; Ishii and Mitsukuri 1960; Khan 1975; Kurata and Omura 1978; Kurata et al. 1981 a; Fukui 1986 a; Chung and Wu 1987). Kurata and Omura (1978) reported the complete identification of somatic rice chromosomes and numbered the rice chromosomes based on the length order. Identification of meiotic rice chromosomes has also been carried out (Shastry et al. 1960; Khush et al. 1984; Chung and Wu 1987; Chen et al. 1982; Wu et al. 1985; Kurata et al. 1981 b). Khush et al. (1984) successfully identified all the pachytene chromosomes by using a complete primary trisomic series of rice. There have, however, been several discrepancies in the characterization and identification of the rice chromosomes, not only between, but also within, the meiotic or mitotic chromosomes (Oka and Wu 1988), mainly due to the lack of an objective method for the identification of the rice chromosomes, such as the G-banding method in higher vertebrate chromosomes. Thus, all of the reports relied upon the visual inspection of the photographic image of rice chromosomes. For experienced and skillful researchers, it may be possible to identify all the rice chromosomes (Ishii and Mitsukuri 1960; Kurata and Omura 1978). However, due to individual fluctuations in judgement caused by differences in personal experience, skill, and sensitivity, uniform data could not always be recorded.

We, therefore, employed imaging methods to obtain objective image data that can characterize each rice chromosome. By combining the quantitative image data with numerical parameters of the length and arm ratio acquired with the highest accuracy, we were eventually able to identify all the rice chromosomes objectively and to establish a quantitative cytological map of rice.

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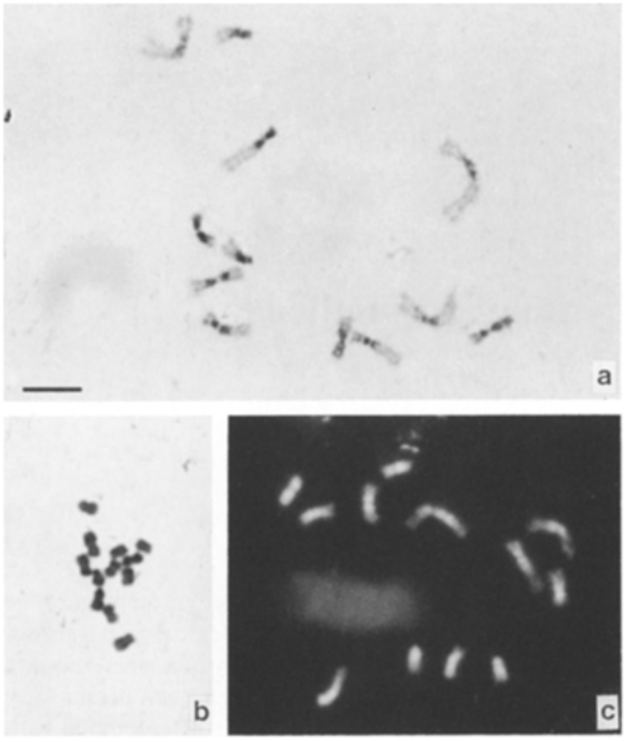


Fig. 1 a–c. Somatic rice chromosomes. **a** Mitotic pro-metaphase rice chromosomes stained with Giemsa. Uneven staining characterizes individual chromosomes. The photograph was exposed under normal conditions. Bar indicates 5 μm. **b** Giemsa-stained chromosomes from a haploid rice plant at mid-metaphase. Rice chromosomes are contracted completely and structural variation could barely be observed. The karyotypes were more or less similar and difficult to discriminate. **c** Pro-metaphase chromosomes stained with ethidium bromide, observed by fluorescence microscope. Fluorescence intensity is uneven along the chromosomes with a mirror pattern of that obtained by the Giemsa stain

Materials and methods

Plant materials and cytology

Haploid plants of the rice variety "Koshihikari" (2n = 12, kindly provided by Profs. H. Niizeki and T. Shimada, Ishikawa Coll. Agr., Ishikawa) were used as materials. Root tips were excised and immediately fixed in a fixative (methanol:acetic acid, 1:1) without any pretreatment. After 1 h fixation, they were washed thoroughly and subjected to enzymatic maceration on a glass slide. Two or three drops of the enzymatic cocktail (4% Cellulase Onozuka RS, Yakult Honsha Co., Ltd., Tokyo; 1% Pectolyase Y-23, Seishin Pharmaceutical Co., Ltd., Tokyo, pH 4.2) were added to an excised root tip on a glass slide, which was incubated in a moisture chamber for 50 min at 37 °C. After the root tips were rinsed, they were subjected to a hypotonic treatment in water for 15 min. They were then tapped with the tip of a fine forceps into small, almost invisible, fragments by adding some drops of a fixative (methanol:acetic acid, 3:1) and then air dried. The glass slides were dehydrated with 100% methanol for 10 min and dipped in the 4% Giemsa solution (pH 6.8) for 30 min, followed by brief washing with tap water.

After complete drying, the preparations were observed under a microscope and the good pro-metaphase chromosome spreads, with a full complement and without any overlapping or heavily attaching sites, were photographed under two different conditions, normal exposure and underexposure. Black and white negative film (Neopan F, ISO 32, Fuji Photo Film Co., Ltd., Tokyo) was used with a combination of the developer (Microfine, Fuji Photo Film Co.). All the photographs were enlarged to a magnification of × 2000 and printed on black and white paper (FM3, Fuji Photo Film Co.). Some of the glass slides were stained with ethidium bromide (5 μg/ml) and observed under a fluorescence microscope.

Analysis of photographic images

The photographic images of the rice chromosomes were digitally stored in a chromosome image analyzing system, CHIAS (Fukui 1985; 1986b; 1988), through a TV camera. Each digital image consisted of a 512 × 512 pixel matrix with 256 steps of the gray value for a pixel. More than 100 digital images from 30 pro-metaphase photographs were subjected to image analysis using the CHIAS. Characteristics of each rice chromosome were extracted from underexposed photographs. Detailed analytical procedures of the rice chromosomes will be presented later in plates of representative imaging steps. Relative length and arm ratios of the chromosomes were also obtained by image analysis of 60 chromatids from the 30 pro-metaphase chromosomes of normally exposed photographs.

An image parameter of the chromosome, CP (Fukui 1989; Fukui and Mukai 1988; Fukui et al. 1988), or density distribution profile of each of the two chromatids of a pro-metaphase chromosome was also digitally determined basically using the underexposed photographs, although information extracted from normal and underexposed photographs of the same chromosomes was combined into a single image by image manipulation at the time of analysis. After the acquisition of all the data for the CPs from 720 chromatids, the standardization for correction of the difference in stainability among chromosomes or among chromosomal spreads was carried out digitally (Iijima and Fukui 1991). Then, 60 CPs that were obtained from 30 chromosomes were averaged by a computer and a standard CP was determined. Based on the standard CPs, and ideogram or a map of each rice chromosome was drawn giving the address within a chromosome.

Results and discussion

Mechanism of uneven stainability and CP acquisition

Somatic rice chromosomes at the mitotic mid-metaphase stage were markedly contracted, being 1–2 μm in size as shown in Fig. 1 b. Since their morphology was similar, it was difficult to identify them. On the other hand, the pro-metaphase chromosomes were two- to threefold longer and their stainability was markedly uneven (Fig. 1 a). Uneven stainability of somatic pro-metaphase chromosomes of rice, which had already been reported with Giemsa staining (Kurata and Omura 1978) and acetoorcein staining (Fukui 1986a), has been employed as one of the key characteristics for the identification of rice chromosomes. The mechanism of the unevenness is not due to preferential stainability or affinity of dyes to specific chromosomal regions, like the G-band in vertebrate chromosomes that appeared after Giemsa staining (Comings 1978), but to the differential

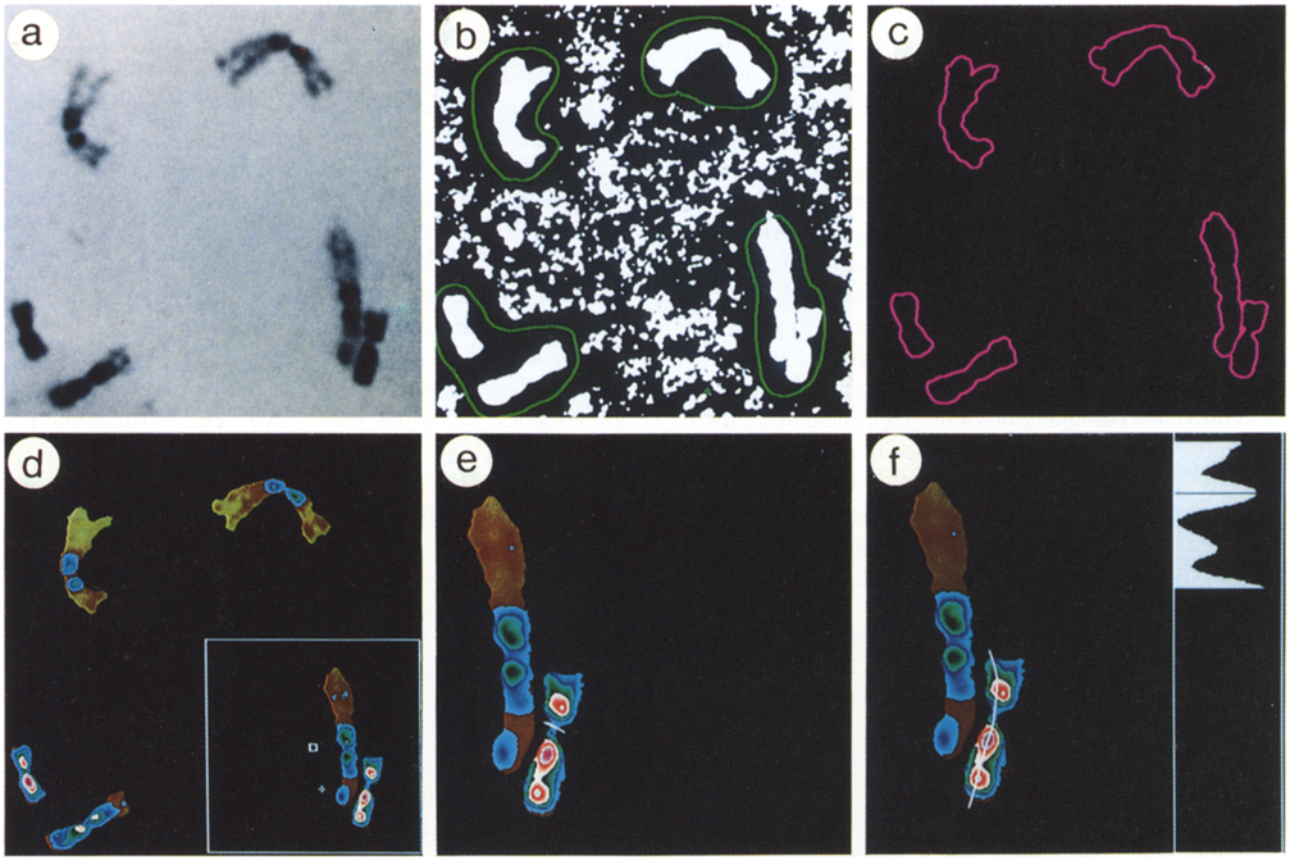


Fig. 2a–f. Representative steps of image manipulation of rice chromosomes by the CHIAS. **a** Original gray image obtained from a normally exposed photograph. The distortion of the density distribution caused by optical systems and illumination of a copy stand was adjusted by shading correction, using a reference blank image. **b** Binarization of the gray image. The image had been cleaned by the application of a median digital filter with a matrix size of 5×5 pixel. The green overlay lines that distinguished between chromosomal and dust regions were drawn interactively using a digitizer. **c** Chromosome contour lines extracted. **d** Combination of the information of the chromosome contour and the digital image from an underexposed photograph, resulting in a chromosome image with a fixed contour and adequate density distribution. The gray image was artificially colored by the look-up-table no. 59 using a CHIAS. **e** Enlargement of the original image. Detailed chromosomal structures could be clearly visualized. **f** Interactive tracing of the chromatin mid-rib with a digitizer gave a CP on a high resolution TV monitor. The numerical data of the CP were simultaneously stored in the CHIAS. Numerical data of short and long arm lengths were also obtained (Kamisugi and Fukui 1990; Iijima and Fukui 1991). They were subjected to various statistical analyses using a personal computer

compactness of the chromatin fibers along the chromosome.

Figure 1c shows the fluorescence staining of rice chromosomes with ethidium bromide. Intensity of the fluorescence is directly correlated with the volume of the DNA at the site, since ethidium bromide molecules emit fluorescence by binding to the double-stranded nucleic acids. There was an obvious unevenness in the fluorescence intensity along the rice pro-metaphase chromosomes, as can be seen in Fig. 1 c. One-to-one correlation of the fluorescent pattern to the Giemsa-stained pattern was confirmed by subsequent Giemsa staining after fluorescence staining of the same sample. Thus, the uneven density distribution appearing in the pro-metaphase chromosomes after the Giemsa staining was ascribed to the differential condensation of the chromatin within a chromosome.

Figure 2 illustrates representative steps of image analysis of the rice chromosomes, to obtain numerical data of the image parameter, CP, and arm ratio and length parameters from both chromatids of a chromosome. Figure 2a shows the original digital image of rice chromosomes from a normally exposed photograph. Distortions caused by illumination and optical systems (e.g., TV camera lenses, microscopic lenses, etc.) were corrected, and the contrast of the photographic image was enhanced by the application of a digital filter. The chromosomal regions were then extracted from the background as white objects, by setting the lower and upper thresholds of the gray values for the original image.

Nonchromosomal regions having the gray values within the thresholds were also extracted as white objects. Interactive discrimination of the chromosomes and dust regions was carried out by drawing a green overlay line and dust was removed (Fig. 2b).

Chromosomal contour lines extracted from the binary chromosome images were obtained from the original gray image to limit the extension of chromosomal regions (Fig. 2c). The use of a normally exposed photograph was found suitable for the identification of the chromosomal contours, where the chromatids at the prometaphase stage appeared as faint extensions. On the other hand, an underexposed photograph was found to be suitable for the detection of the accurate density distribution of the chromosomes. Thus, the information on both the chromosome contour from a normally exposed photograph (Fig. 2c) and the chromosomal density distribution from an underexposed photograph of the same chromosome image was adequately combined into a single digital image.

The image was then subjected to pseudo-coloration with the artificial colors generated by the CHIAS (Fig. 2d). The artificial coloration drastically improved the image so that the actual density distribution within a chromosome could be easily characterized by human vision. The most condensed region in the figure was distinctly displayed by a dark pink color. In three larger chromosomes, the absence of the pinkish region clearly demonstrated the lack of any heavily condensed region, which in three shorter chromosomes were demonstrated by the presence of the color. One of the chromosomes in the right corner had three condensed regions along the chromosome, which were clearly visualized by the difference in color.

For the acquisition of the numerical data of the CP, the original figure was enlarged twofold so that the line could be drawn more easily. A centromeric line was superimposed onto the primary constriction, which appeared as an isthmus between the short and long arms (Fig. 2e). After writing down the overlay centromeric line into the chromosome image with the gray value 0, the mid-rib of a chromatid was manually traced (Fig. 2f). The CP of the chromatid was displayed in the box in the right corner. A horizontal line at the top of the graph showed the gray value and a vertical line on the left side showed the number of pixels. The lower gray value or a concave of the profile showed the heavier condensation of the chromosome. The centromeric position was depicted as a horizontal black line around the central region of the profile line. The upper side of a centromeric line corresponded to the density distribution of the short arm, and the lower side of the centromere, to the density distribution of the long arm.

The visual recognition of the three condensed regions was now replaced by the three concaves on the CP along the chromosome and the data could be obtained digitally. Length parameter was also measured based on the length of the mid-rib of each chromatid.

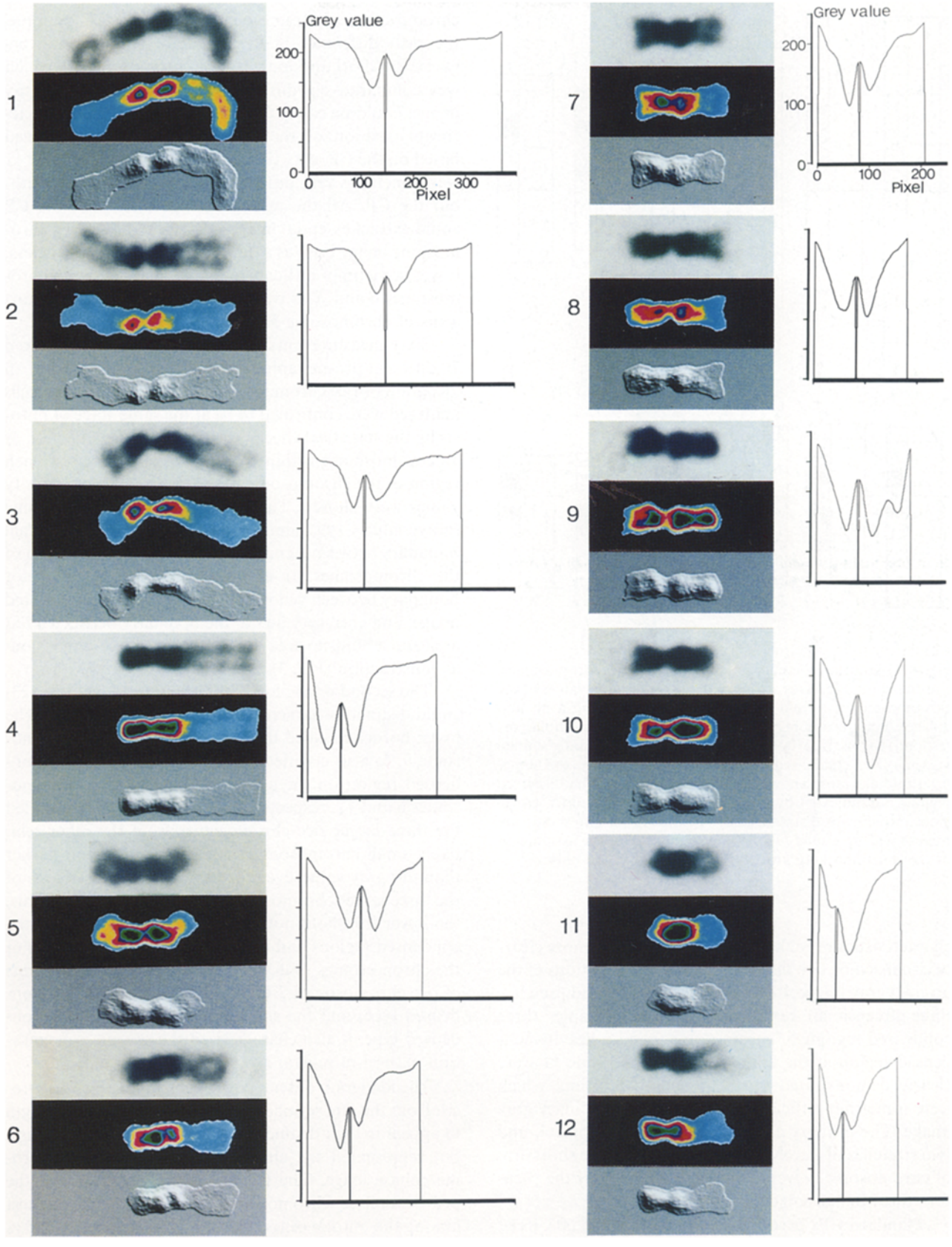
An automatic mode for the image processing, with the exception of a few interactive steps, ensured the reproducibility and exact quantitation of the image information of rice chromosomes. Various imaging techniques could guarantee the accuracy and reproducibility of the analysis, even in the process of manual operation, by complementing human visual recognition; that is, pseudo-coloration clearly demonstrated the extension of the telomeric part of the chromosomes, stretch and overlap of each chromatid within a chromosome, the mid-rib of a chromatid, and the location of the centromere. Digital filtering for the enhancement of the contrast and digital enlargement of the image were also very effective making it possible for minute chromosomal structures to be recognized easily by human vision. Moreover, although the parameters of the length and arm ratio could be measured by conventional methods, it was only possible to obtain the gray values by imaging methods.

A stable pattern of the chromosomes was maintained in terms of numbers of CPs since the standard errors of the 60 CPs were negligible, suggesting that each rice chromosome retained a characteristic pattern repeatedly during the pro-metaphase stage at mitosis. Thus, it was concluded that the CP could be a good image parameter for characterizing the dynamic patterns of rice chromosomes.

Identification of rice chromosomes based on the CP

Figure 3 illustrates the rice chromosome images analyzed by imaging techniques. The standard CPs for all the rice chromosomes are also presented. Rice chromosomes were arranged according to their length order. At the top of the panel, original chromosome images are given. The

Fig. 3. Somatic rice chromosome aranged in order of length. Numbers of the *left side* of the chromosome panel are based on the average total chromosome length. The *top image* of the panel indicates the gray image of the ordinary Giemsa-stained chromosome. The *middle image* demonstrates the pseudo-colored or artificially generated color image by the look-up-table no. 57 of the CHIAS. The *bottom image* shows the pseudo-, three-dimensional representation generated by a pseudoplast digital filter treatment of the original image (Fukui and Ito 1989). The standard CPs are presented on the *right-hand* side of the chromosome panel. The vertical axis indicates the gray values; the higher the gray value, the lower the density. The horizontal axis represents the number of pixels from the end of a short arm to the terminal of a long arm. All the morphological characteristics of the rice chromosomes could be clearly visualized by these four different representations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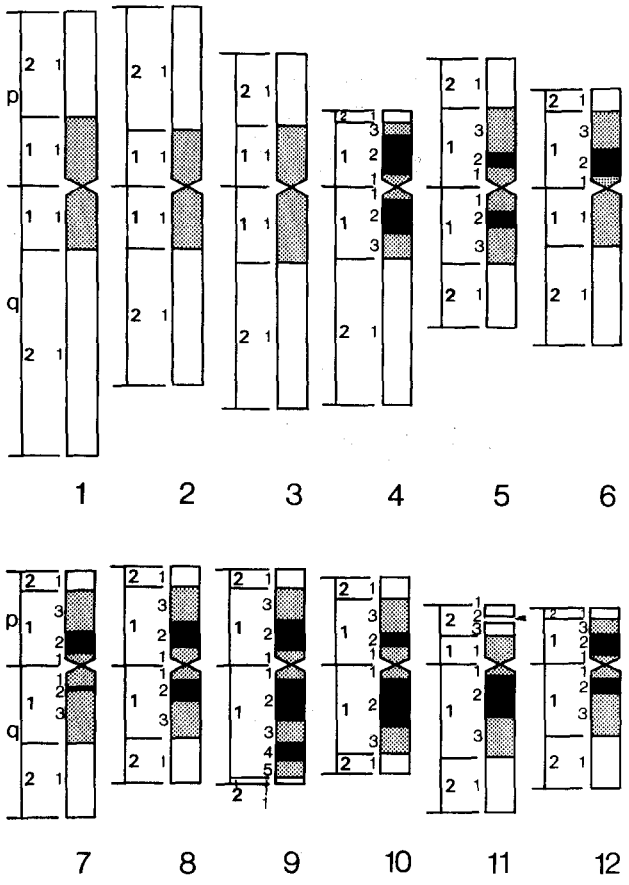

Fig. 4. Quantitative ideogram of somatic rice chromosomes. Chromosome addresses are given based on the gray values of the chromosome. *p*, *q*, and ◂ indicate short arm, long arm, and nucleolar organizing region, respectively. Upper threshold corresponds to the visual boundary of the darkly and faintly stained regions. The condensed regions discriminated by the upper threshold are represented by the *dotted regions*. The dispersed regions are indicated by a blank bar. The most condensed regions, which are distinguishable by the lower threshold, are represented by a *solid box*. For chromosome 5, the assignment of the short and long arm was based on its length only

characteristics of each chromosome could be more clearly visualized by two different digital manipulations of the original gray image, i.e., pseudo-coloration and pseudo-, three-dimensional representation. For example, three condensed regions of chromosome 9 and a heavily condensed region at the long arm of chromosome 11 were clearly demonstrated by the pseudo-coloration, which were somewhat difficult to visualize in the ordinary gray image. The primary constriction of chromosome 6, and two stretches of the chromatid terminals at the short arm of chromosome 9, were typically visualized by the pseudo-, three-dimensional representation.

Standard CPs based on the average of the 60 CPs of each chromatid also characterized the degree and location of the condensation regions in the pro-metaphase chromosomes in a more convenient way. For example, the clear difference in the degree of condensation between the short and long arm of chromosome 10, which was comparatively difficult to distinguish on the gray image, could be easily demonstrated by the CP. The accurate position of the centromere was also obtained based on the CP, e.g., the centromere of chromosome 11 was sometimes very difficult to localize objectively without the CP. All the results demonstrated that the CP could extract essential image information from the chromosome image itself as a digital parameter. Nevertheless, it was sometimes difficult to discriminate between chromosomes 6 and 7, as well as between the long and short arms of chromosome 5.

Sixty measurements of the length and the CPs from 30 different pro-metaphase plates were represented in an ideogram of rice chromosomes (Fig. 4). Thirty of the cells analyzed were confirmed to be at the same stage of mitosis by the statistical check ($\alpha = 0.01$) of the total length of all chromosomes within a cell. In order to analyze each region of the chromosome based on the CP, two density values were chosen. The upper or lighter density value (gray value = 199) almost corresponded to the visual boundary between a condensed and a dispersed region of the chromosomes. It was also indicated as the color boundary between yellow and blue in the pseudo-colored image, and corresponded to the boundary between a flat area and a built-up area in the pseudo-, three-dimensional construction (Fig. 3).

The second or the lower threshold (gray value = 123) could distinctly characterize each rice chromosome. The lower border revealed three concaves unique to chromosome 9. It also clarified a peculiar, single, heavily condensed region on the short and long arms of chromosomes 6 and 11, respectively. Moreover, it discriminated the three larger rice chromosomes from the other relatively small chromosomes. There was no region darker than this gray value, even for the most condensed area of the three larger chromosomes in the standard CPs. Thus, the lower threshold not only discriminated between the condensed regions and the heavily condensed regions of the chromosomes, but also between two different kinds of rice chromosomes, i.e., the large, empty, and late condensed type, and the small, substantial, and early condensed type; it also characterized the specific condensation pattern of several rice chromosomes.

The ideogram did not cover the whole stages of mitosis, from the late prophase when the chromosomes begin to appear in their distinctive shape to the mid-metaphase, but represented the chromosomal pattern of the pro-metaphase stage. The frequency of appearance of the pro-metaphase chromosomal spreads was the highest among the mitotic cells observed. It amounted to 47.5% of total dividing cells when no pretreatment was applied in rice root meristematic tissues (Iijima and Fukui 1991).

Table 1. Numerical data of somatic chromosomes of rice[a]

Chromosome no.	Relative length *,[b]	Arm ratio *,[b]
1	13.6±1.1	1.58±0.23
2	11.7±1.2	1.10±0.25
3	10.9±0.9	1.72±0.21
4	9.1±0.6	2.93±0.29
5	8.3±0.6	1.12±0.09
6	7.9±0.6	1.68±0.21
7	7.6±0.5	1.61±0.31
8	6.6±0.4	1.24±0.13
9	6.6±0.5	1.26±0.11
10	6.1±0.4	1.26±0.12
11	5.8±0.6	3.90±1.11
12	5.8±0.4	2.00±0.37

[a] Average data of 30 pro-metaphase plates with a total chromosome length ranging between 39.7 and 54.4 μm
[b] Length of the satellite was excluded from the calculation
* Standard deviation

In other words, the pattern modeling in the ideogram covers the longest period among the mitotic stages. The ideogram is also a quantitative cytological map developed for the first time for somatic rice chromosomes. Every fractional length of each part of the chromosome determined by two thresholds of the density was quantitatively decided. Therefore, the chromosome map constructed here depicted not only the most common rice chromosome features, but was also the numerical basis for the characteristics shown in the ideogram, i.e., fractional length, fractional area, average density of the region, occurrence frequency of the traits, etc. It may also serve as a valuable tool for the identification of each rice chromosome upon visual inspection of the chromosomal specimen, since the ideogrammic pattern presented here resembled the visual pattern.

Table 1 presents numerical data of the somatic chromosomes of rice. Chromosome numbers correspond to the numbers indicated in Fig. 3. The total length of 30 complements ranged from 39.7 to 54.4 μm and the values were not statistically significant ($\alpha=0.01$). It was also demonstrated that there was no significant statistical difference ($\alpha=0.01$) between the chromosomes in the pairs of chromosomes 2 and 3, 5 and 6, 6 and 7, 8 and 9, 10 and 11, and 11 and 12. Differences between chromosomes 2 and 3 and between 6 and 7 were significant at the 5% level. Since the difference between neighboring chromosomes is so small, it is likely that there will be discrepancies among the results on the number of rice mitotic chromosomes. The occasional fluctuations associated with the limited number of chromosomal spreads analyzed and the difference in the pattern of each rice chromosome along the mitosis may account for the differences in the results. The results presented here are consistent with the numbering system established by Kurata and Omura (1978), if chromosomes 2 and 3, 5 and 6, 8 and 9, and 10 and 11 replaced each other within the pairs. The differences in length between the paired chromosomes were all statistically nonsignificant ($\alpha=0.01$).

Utility of image analysis methods in chromosome research and value of the chromosome map of rice

The CP analysis using imaging methods has already been reported to be effective for the identification of atriplex chromosomes that are also very small (Fukui and Mukai 1988). The CP, i.e., the uneven condensation at the pro-metaphase stage along the chromosome, has commonly been observed in small plant chromosomes such as those of *Nicotiana otophora* (Merrit and Burus 1974), tomato and *Brassica* spp. (Murata and Orton 1984), soybean (Yanagisawa et al. 1990), etc., but not in the higher vertebrate chromosomes, nor in plant chromosomes with a larger size such as those of wheat, barley, rye, etc. Appearance of uneven condensation may depend on the differences both in the number and location of the contraction centers of the chromosomes. Chromosome 9 may have three condensation centers. The usefulness of the CP in identifying rice chromosomes may be associated with its stability. The basic pattern of the CP for the chromosomes appeared reproducibly.

Imaging methods employed in the analysis have been an indispensable tool for the acquisition of exact quantitative data, as they complement human visual recognition by various means. Moreover, once the program is constructed, they are not technically demanding, nor is the analysis time-consuming. Presently, a period of about 10 days may be sufficient to obtain all 720 CPs from 30 haploid complements, which correspond to all the data used in this report. These methods are not only efficient in obtaining the numerical data, they also make it possible to acquire data that could not be obtained by conventional methods, such as the CP.

The ideogram was not based on the mid-metaphase chromosomes but on the pro-metaphase chromosomes. Since the size of the rice chromosomes at the mid-metaphase stage ranges from 1 to 2 μm, chromosome maps at this stage are usually too simple and are thus insufficiently detailed for chromosomes to be distinguished. Furthermore, no banding method has been developed to identify each mid-metaphase chromosome. The chromosome map constructed here has the following advantages: first, the pattern of the ideogram corresponds to the visual pattern of the actual pro-metaphase chromosomes; second, the pattern represents the most frequent chromosomal patterns or covers the longest period of mitotic metaphase stage from the pro-metaphase stage to the mid-metaphase stage; third, all the data shown in the ideogram have a numerical basis, as image analysis methods were employed throughout the study.

The chromosome map based on the CP is therefore considered to be not only useful for the identification of rice chromosomes, but also fundamental to cytological, genetic, and biotechnological studies on rice.

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