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Standardization of karyotyping plant chromosomes by a newly developed chromosome image analyzing system (CHIAS)

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Summary. A chromosome image analyzing system (CHIAS) has been developed especially for plant chromosomes. A standard karyotyping method using CHIAS is also described. The characteristics of the CHIAS are as follows: 1) the main objects of the analysis are plant chromosomes, 2) it constructs a man-machine interactive system to put researchers' decisions into the analytical process, 3) it can automate the routine part of an analysis as much as possible, and 4) it digitizes the image information of chromosomes and analyzes them. Software for karyotype analysis of plant chromosomes has been developed. Thus, in the case of rye chromosomes, it is possible to get quantitative data for all chromosomes and a karyogram within 25 min.

Key words: Chromosome – Karyotyping – Rye – Image analysis – Computer system

Introduction

Image analyses of chromosomes have almost two decades of history if we take the most primitive methods into account. Most of the studies in the early stages concentrated mainly on the quantification of the density of stained chromosomes picked up directly through a microscope or recorded on a photograph by densitometric equipment (Carlson et al. 1963; Mendelsohn et al. 1966).

Analyses of the banding patterns of chromosomes were also attempted (Caspersson et al. 1971; Caspersson et al. 1971; Lubs and Ledley 1973; Marimuthu et al. 1974; Mason et al. 1975). In the mid 1970s, a trial model for fully automated karyotyping, including the automatic preparation of the slides, was developed for human chromosomes (Castleman and Melnyk 1976). Both the accumulation of methodologies for automatic karyotyping of human chromosomes and the recent rapid development of microelectronic techniques (e.g. Fujita 1983) make it possible to analyze images of human chromosomes automatically (Lundsteen et al. 1980; Taylor and Graham 1980).

For plant chromosomes, however, there have been almost no reports on the image analysis system, excluding a simple densitometric system or a TV monitoring system. A possible technical reason could be as follows: the system for plant chromosomes would be required to analyze the chromosome set of four to more than a hundred in number in order to meet the demands of researchers. Plants have a wide variety of chromosome numbers and researchers often do not restrict their interest to only one species. The existence of polyploidy and aneuploidy, which are frequently found in many species and even created artificially, makes a system architecture more and more complicated. Therefore, simple standardization, especially in software, could not be attained for number and size of chromosomes to be analyzed.

From the point of view of the preparation procedure, plant cells are not as good study material as animal ones. Their hard cell walls sometimes prevent a good spread of the chromosome set on a glass slide. Pretreatment methods, such as HCl treatment and enzymatic maceration, have not improved the situation drastically. Moreover, a lower synchronization of cell cycles than animal cells is found even when such methods of pretreatment as low temperature, colchicine, 8-hydroxyquinoline, etc are used. Bias in the photographic image is caused by every procedure of taking a picture: development, enlargement and printing. These fluctuations are very difficult to control and make it difficult to get reproducible results. Data fluctuation originating in photographic and measuring processes sometimes result in significant differences even in the same material. Therefore, the chromosome study of plants requires much experience and patience even for the most experienced researchers. A more flexible architecture of the system, both in software and hardware, is also necessary relative to the case of animal ones.

The quantification of the image data of chromosomes has been almost impossible except for some simple parameters by ordinary means: the length of the short and long arms, and their ratio represent almost all the chromosome image information now available.

These parameters, however, are not very accurate in identifying plant chromosomes in many cases. We need a system which can objectively, easily, and speedily provide quantitative chromosome image data based on fixed procedures. Devices of microelectronics and image analysis methods have been developing rapidly in these years and now they can supply indispensable tools for developing an image analysis system for plant chromosomes.

I describe here a chromosome image analyzing system (CHIAS), which has been developed using commercially available units. I also present a standard karyotyping method of plant chromosomes using this system.

Material and methods

Chromosome images analyzed

A photograph of C-banded rye chromosomes was used as an example of a chromosome image medium. It was black and white, 10×10.5 cm in size, and kindly provided by Dr. Takashi Ryu Endo, Nara University, Nara, Japan.

Design and architecture of the CHIAS

The following conditions are considered to be indispensable in developing the system (Fukui 1985). 1) The main objects to be analyzed by the system are plant chromosomes. Therefore, the system must have the versatility and capacity to handle chromosomes varying from four to 120 in number. 2) Importance can be attached to the decisions of experienced researchers in the analyzing process. So it must be a man-machine system. In other words, we can participate or interact in the necessary steps during the chromosome analysis. 3) Routine work in image processing should be automated as much as possible. Therefore, the system can process the programmed procedures automatically when the researchers' decision is not invaluable. 4) An attached microscope in the system can be operated either manually or automatically. Operation of a microscope usually demands the manpower of a researcher during the ob-observation. Therefore, the system must have automatic fosucing and scanning functions, which occupy most of the operational actions. 5) As most of the chromosome information has been stored in photographs so far, I used a photograph as the original medium of chromosome image information in this paper. Photographic processes, however, are one of the most variable part of image processing. It depends greatly on the person and is difficult to control in getting reproducible results. Therefore, the system has to have the ability to obtain chromosome information directly from a microscope through a television camera. Digitization of image information is necessary to manipulate image information mathematically and to use digital equipment as the storage medium.

Taking all the conditions mentioned above into account, a microscope and its units for automation, units for data input, units for image analysis, and units for data output and storage were selected among commercially available equipment, and integrated.

A fluorescence microscope (Photomicroscope III, Zeiss, Oberkochen) with autoscanning stage (movable area 50×75 mm with a pitch of 0.1 μm, Gebr. Märzhauser Wetzlar OHG, Steindorf) and autofocusing unit (Motor Adapter MA4, Gebr. Märzhauser Wetzlar OHG) was chosen. A photometer (01K, Zeiss) and a filter system (Continuous filter monochromater b, Zeiss) are also attachable as the need demands. A high resolution TV camera (CTC-2600P, Ikegami Tsushinki Co., Ltd., Tokyo) was directly mounted on the top of the microscope. Images on the photographic papers or films can be taken through a zoom lens (Fujinon C6×17.5B, Fuji Photo Optical Co., Tokyo) and a close-up lens (Fujinon CL8658, Fuji Photo Optical Co.). Image analysis units (IBAS I, II, Kontron/Zeiss) were selected as the image processing component of the system. For output of the data and images, a printer (DP-250, Oki Electric Industry Co., Ltd., Tokyo) and a color image recorder (CIR-100, Nippon Avionics Co., Ltd., Tokyo) were chosen. A videotape recorder (AG-6200 ENZ, National, Osaka) was also chosen as an additional input/output unit.

Software for general image analysis was provided by Kontron/Zeiss and some of the basic commands for chromo-

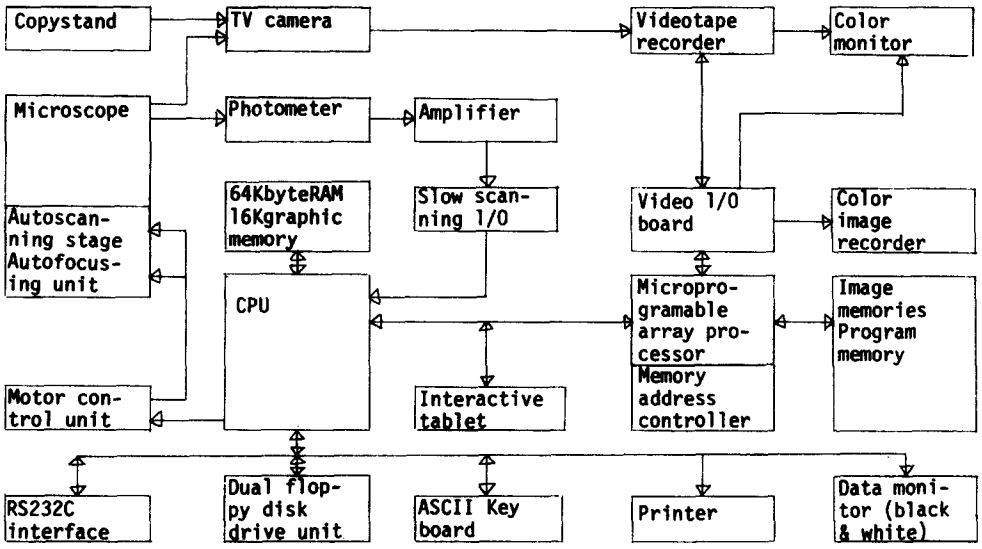


Fig. 1. Block diagram of the CHromosome Image Analyzing System (CHIAS)

some analysis were developed by Kontron based on my software design. A software for karyotype analysis was programmed using these general and basic commands.

Results and discussion

Flow of chromosome information

Figure 1 shows a block diagram of the system with flow of commands and chromosome information.

After allocation of an ID number of the measurement, ten photographic images of an original photograph were taken through a TV camera and an averaged digital image was stored in an image memory. It took 40 ms to get each digital image. Taking an average image significantly reduces random noise in the resulting gray image and improves the S/N ratio. One image frame has a 512×512 of pixel matrix and each pixel has 8 bits of information (256 gray-level value). Thus, each image frame requires 256 K bytes of memory size.

A digitized image was then subjected to several image manipulations automatically and manually by a keyboard or a cursor operation. All the outputs of numerical data are displayed on a black and white data monitor and printed on the printer. An 8 in floppy disk is also used for the storage of numerical data. The output of an image is displayed on a color monitor, stored by a color image recorder, or dumped on a printer by request. An 8 in floppy disk and a videotape are also used for the storage of image data. It took about 1 min to dump a whole image frame on a floppy disk and about 2 min to print.

Software for the karyotyping of plant chromosomes

Software for the karyotyping of plant chromosomes recorded on the black and white photographs was constructed. It is divided into two sub-programs. Figure 2 shows the flow charts of them. The former is the program for acquisition of quantitative data from each chromosome and the latter is for karyotyping, that is, placement of each chromosome in order of length or chromosome number, etc. Needless to say, a well spread metaphase image results in obtaining more reliable data and saves analysis time, although CHIAS can also analyze overlapped and touching chromosomes.

The first group of commands is the preparatory steps for making an original digital image for subsequent analysis. An averaged digital image of ten images is created as an original gray image (Fig. 3 a). Shading correction is necessary to obtain a good image since there is always some irregularity in the image caused by irregular illumination of a copystand, and deflection,

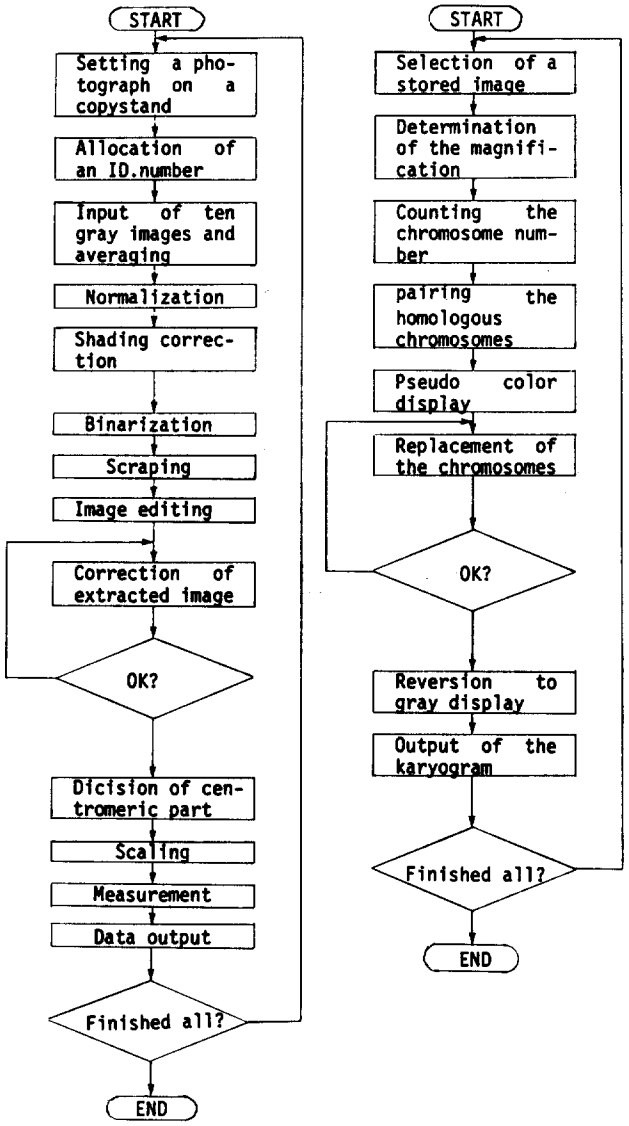
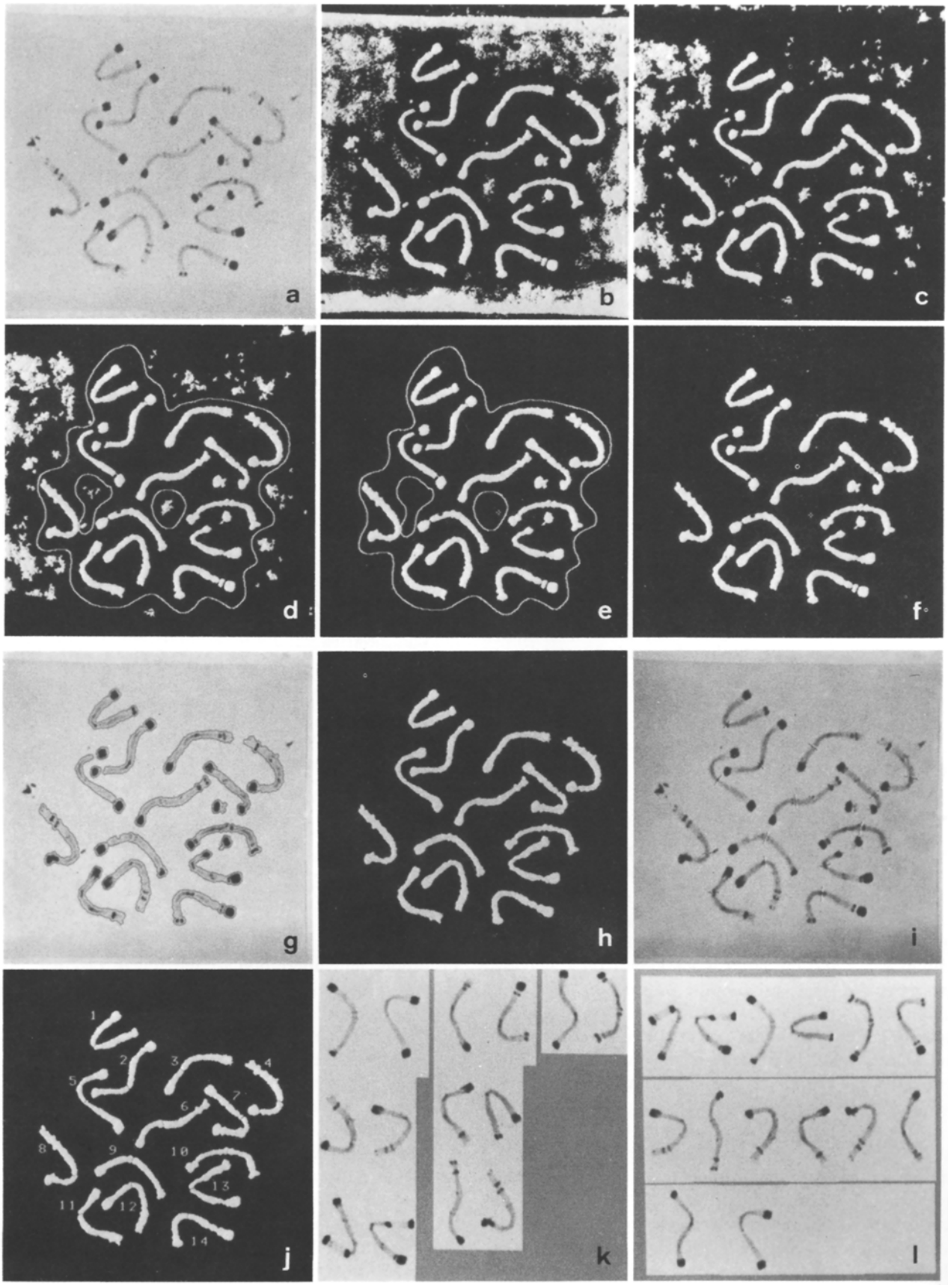


Fig. 2. Flow charts for data acquisition and karyotyping of plant chromosomes (ver. 2.2)

distortion, and aberration of the image pick-up apparatus. Fluctuation and noises as a result of uneven staining of the preparate and illumination of the microscope sometimes cause serious bias in the gray image. The above problems are, however, very difficult to correct in the case of photography. On of the reasons is that there is no reference image for a given photograph. Therefore, several digital filters which generate artificial reference images for the shading correction of those biases were tested and a lowpass filter was selected. In this case this method of shading correction proved to be enough to discriminate good chromosome images from the background. The size of the matrix of a coarse lowpass filter was significant in creating a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proper reference image. A filter matrix of 60×60 pixel square was decided empirically as the most suitable one.

Another preparatory step is the enhancement of the image contrast by mapping the original gray levels of the image linearly to the maximum range of 0–255 of the gray levels. Deletion of randomly distributed noises which had arbitrary gray levels was necessary to accomplish this. The threshold of the noise frequency to be eliminated was set as one and was found to be sufficient to obtain a good enhanced image. This step was inserted just before the artificial shading correction.

The second group of commands is for a proper extraction of each chromosome image from the background, which corresponds to cutting out the chromosome images from a photograph by scissors in ordinary karyotyping. For this purpose I used a method of pointing the lower and upper thresholds of gray levels of the image and extracted the range of gray levels between them as the chromosomal levels. The first time it was performed manually in order to decide the proper lower and upper limits of the gray level. A binary image, where the chromosomes were shown as white (gray level = 255) and the background as black (gray level = 0), was then produced automatically. Figure 3 b shows the result of chromosome discrimination. Shading correction and binarization are actually involved in one command which executed both steps sequentially. As a result, all the chromosomes were extracted adequately but there were also many "dust" particles like sprinkled salt in the background. A digital filter for scraping off the white ones having an area smaller than ten pixels and larger than 3,000 pixels eliminated most of the dust (Fig. 3 c). There still remained, however, many dust particles having a size between the set conditions and some of them were similar to the chromosome area. As the same digital filter could not be used for these dust particles without risking the elimination of some chromosomal parts, another method had to be adopted. Figure 3 d shows the editing of the binary image by selecting the necessary area using overlay lines controlled with the interactive tablet and the cursor. Once it was set, the dust particles were easily eliminated by pointing to the unnecessary area with the cursor (Fig. 3 e). Erasing the overlay lines resulted in a good chromosome image with a clear background (Fig. 3 f).

The third group of commands is verification and checking of each discriminated chromosome image. Firstly, a contour line for each chromosome was generated according to the binary chromosome images and was superimposed onto the original gray chromosome images in color (Fig. 3 g). This process is indispensable in carrying out a delicate and exact adjustment of the extracted binary chromosome images based on the original gray ones. If necessary, the binary chromosome images were then modified by image editing. The final binary image was obtained after a comparison using the contour lines of the adjusted chromosomes and the original gray image (Fig. 3 h). Much less time for adjustment will be required when uniformly stained chromosomes are used as the objects of investigation.

The fourth group of commands is the determination of centromeres and data acquisition. The centromeric part was determined interactively for each chromosome using a superimposed line which was drawn on the gray image by the cursor (Fig. 3 i). These color lines showing the centromeres were then copied with the gray level 0 onto the binary image, which meant separation of a long and a short arm at the same time. The identification number was generated for each chromosome (Fig. 3 j). Scaling for the determination of the unit of measure was done before calibration. Two parameters, such as area and perimeter, and also the ratio of these parameters between the short and long arms were calculated based on this binary image. The length parameter was of almost no use in automatic calibration since detection of a midrib for each chromosome is sometimes impossible without the help of the experienced researchers' knowledge. Therefore, interactive mode was adopted for drawing midribs on chromosomes and their length and the ratio of the short and long arms were calibrated. Numerical data of each chromosome were given on the data monitor and the printer.

For karyotyping, the enhanced gray image (Fig. 3 a) and the binary image (Fig. 3 h) obtained in the previous process were employed. Adjustment of the size of the chromosomes to the karyotyping frame could be performed in this step depending on the study demand.

Fig. 3a–l. Results at each step of image manipulation of rye chromosomes by CHIAS: **a** Original gray image; **b** Binarized image after lowpass filter treatment; **c** Elimination of large and small dusts; **d** Discrimination between chromosomal and dust area by overlay lines drawing on the binary image; **e** Elimination of the dust by cursor pointing; **f** Binary image with clear background; **g** Comparison between original gray image and extracted binary chromosome image by overlapping of contour lines of binary images on the original gray image; **h** Modified binary image; **i** Decision of the centromeric part for each chromosome by overlay line drawing; **j** Separation of the short and long arm and numbering the chromosomes; **k** Paired chromosomes; **l** Karyotyping of rye chromosomes

After counting the chromosome number, the pairing of homologous chromosomes was carried out automatically, based on the maximal diameter of each chromosome. It is, however, not so good in many cases and needs a researchers' aid in order to accomplish it satisfactorily. The paired chromosomes were then displayed on the color monitor (Fig. 3k). A suitable karyotyping frame for any plant species having a chromosome number from four to 120 can be generated using the software in this system. A special frame for karyotyping rye chromosomes was generated beforehand and stored in the system. All the rye chromosomes were automatically arranged in the order of maximal diameter. A pseudocolor display, which corresponded to the actual gray levels, was selected to adequately emphasize the banding pattern of the chromosomes. Misarrangement of the chromosomes could be easily detected under these conditions and replacement was accomplished by the cursor pointing to the required chromosome on the color monitor. The final karyogram of the rye chromosome was thus acquired and the pseudocolor display was reverted to an ordinary gray display (Fig. 31). The karyogram obtained was dumped either on the printer, a floppy disk, a videotape or a photographic film through the color image recorder.

The time required to complete karyotype analysis was about 25 min in this case when the automatic executive mode of the system was adopted: 13 min for the first part and 12 min for the latter. Although the necessary time would depend much on the individual quality of the chromosome plate to be analyzed, CHIAS could save much time compared with ordinary karyotyping procedures. In this case we used a photograph for the original medium of a chromosome image. CHIAS can also take chromosome images directly through a TV camera mounted on a microscope. No photographic and dark room manipulation is needed throughout the analysis. It can, therefore, greatly reduce not only the time but also the efforts of researchers.

This article reports the development of a new image analyzing system for plant chromosomes, CHIAS, and the standardization of the karyotyping procedures using it. This system achieved a drastic reduction of researchers' time and efforts while maintaining a high standard of information quality in the quantification and karyotyping of chromosomes. The development of software of the system is to be continued and the automated searching of metaphase plates by autoscanning and autofocusing will be possible in the near future, even in plant materials.

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