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# High resolution bands in maize chromosomes by G-banding methods

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**Summary.** It was demonstrated that G-bands are unequivocally present in plant chromosomes, in contrast to what had been formerly believed by plant cytologists. Maize chromosomes prepared by an enzymatic maceration method and treated with trypsin or SDS showed clear G-bands spreading along the chromosomes. The most critical point during the G-banding procedures was the post-fixation with glutaraldehyde solution. Banding patterns were processed by using the chromosome image analyzing system and a clearer image was obtained. G-banding technique and the image manipulation method described here can be applied to many plant species, and would contribute new information in the field of plant cytology and genetics.

**Key words:** Maize – Chromosome banding – G-banding method – Enzymatic maceration – Image analysis

## Introduction

Chromosome studies in higher plants had been keeping pace with similar studies in higher vertebrates until the early 1970s when the G-banding technique was developed in human chromosomes (Sumner et al. 1971). The development of the G-banding method made it possible for the first time to identify human chromosomes and to apply this information to the diagnosis of human genetic diseases. More than a thousand molecular markers and genes have also been mapped on chromosomes based on the fine G-banding pattern (McAlpine et al. 1987). Chromosome studies in plants had made little progress in the G-banding method or fine mapping of chromosomes, although some other banding methods, e.g., C-, N-, fluorescence banding, etc., became available for the analysis of plant chromosomes. When these banding methods are used, it is still difficult to identify all the chromosomes in plant species, due to the coarseness in the pattern of the bands. In the case of wheat, whose chromosomes have been one of the most popular cytological targets, 42 chromosomes were eventually identified 1986 by Endo (1986).

G-banding patterns comparable to those in higher vertebrates, however, have not been demonstrated in plant chromosomes for more than 15 years despite the efforts of plant cytologists. Whenever plant cytologists have claimed that they could demonstrate G-bands in plant chromosomes, in many instances these represented uneven contracted patterns of chromosomes observed at the late prophase or early metaphase in mitoses appearing under specific conditions and in particular materials. The banding pattern could not be reproduced even by the application of identical techniques. As a result, it has been considered that G-bands do not exist or cannot be observed, even if they do exist, by high contraction of the metaphase chromosomes in plants for more than 10 years (Greilhuber 1977). A high resolution and reproducible G-banding method for plant chromosomes, however, has been required in both basic and applied fields and studies on G-banding methods were carried out in several laboratories (Chen et al. 1986; Fukui et al. 1986; Zhu et al. 1986).

In 1985, we obtained a clear G-band-like pattern in the chromosomes of *Atriplex patula* L. (Fukui et al. 1989). Based on this result, we started to develop a reproducible and universal G-banding method for plant chromosomes in testing various procedures in several crops.

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In this report, the presence of G-bands in maize chromosomes comparable to those detected in higher vertebrates was confirmed, and the G-banding technique developed for plant chromosomes is presented. The G-banding pattern was digitally manipulated by the chromosome image analyzing system, CHIAS (Fukui 1985, 1986, 1988), and a clearer image was obtained.

## Materials and methods

*Plant material and slide preparation*

A maize (*Zea mays* L.) inbred line, Oh 43, was used. Seeds germinated in small pots filled with moist vermiculite were grown for about 2 days at 32 °C under continuous illumination. Three root tips about 1 cm long were excised from each seed and pretreated either with a 0.05% colchicine solution or 0.05% colchicine solutions containing either 10 ppm actinomycin D or ethydium bromide for 2 h at 25 °C for the G-banding. For the C-banding, the root tips were pretreated with distilled water at 0 °C for 20 h. In the case of G-banding, root tips were subsequently dipped in the Ohnuki's hypotonic solution (55 m*M* KCl, 55 m*M* $NaNO_3$, 55 m*M* $CH_3COONa$, 10:5:2; Ohnuki 1968) for 30 min to 1 h at 25 °C. Then they were fixed with methanol-acetic acid (3:1) for 1–4 days in a freezer (–20 °C) or for at least 2 h at 25 °C to actinomycin-D-pretreated ones. Fixed root tips were subsequently smashed and smeared with a tweezer in dropping fresh fixative, or were macerated enzymatically. For the enzymatic maceration, the fixed root tips were washed for about 10 min and were macerated in an enzymatic mixture (pH 4.2) containing 2% Cellulase RS (Yakult Honsha Co., Ltd, Tokyo) and 2% Macerozyme R-200 (Yakult Honsha) for 20–60 min at 37 °C in a 1.5-ml Eppendorf tube. After rinsing with water two or three times, each macerated root tip was picked up onto a glass slide using a Pasteur pipette, and was cut into small pieces with a sharp-pointed tweezer with the addition of fresh fixative. Glass slides were observed under a phase contrast microscope, and those showing well-spread chromosomes were selected and air-dried for about 2 days in an incubator at 37 °C.

*Banding methods*

The samples prepared by the actinomycin D pretreatment method were directly stained in 10% Wright solution diluted with 1/15 *M* phosphate buffer (pH 6.8) for 10 min at 25 °C. Then they were washed and air-dried. The enzymatically macerated samples were again fixed in a 2% glutaraldehyde solution diluted with the phosphate buffer for 10 min at 25 °C and washed. Then the post-fixed slides were immersed either in 2% trypsin (Merck, Art. 8367) dissolved in PBS (pH 7.2) for 10 min at 25 °C, or in 0.02% SDS dissolved TRIS-HCl buffer (20 m*M*, pH 8.0) for 2–5 min at 25 °C. After the treatment, the slides were briefly washed and air-dried. They were stained in 5% Wright solution in 1/30 *M* phosphate buffer (pH 6.8) for 5 min.

In order to identify knob constitutions in the maize line, the slightly modified C-banding technique of Chow and Larter (1981) was applied to metaphase plates. Slides air-dried at room temperature within a week after preparation were immersed in 5% $Ba(OH)_2$ for 6 min at room temperature and were quickly transferred to 0.1 *N* NaOH for 30 s at room temperature. Then the slides were directly dipped in 2 × SSC for 1 h at 60 °C. After washing, the slides were stained with 2% Wright solution for a period of 30 min to 1 h. Well-banded chromosomal plates were photographed using the Neopan F black and white film (ISO32, Fuji Photo Film Co., Ltd., Tokyo).

*Image analysis*

Enlarged photomicrographs were analyzed by the CHIAS. Slightly underexposed prints were suitable for the image analysis of the G-banding pattern. Each photomicrograph was picked up into the CHIAS by a high resolution TV camera mounted on a copy stand. The image was stored in an image frame memory (512 × 512 pixel matrix within 256 gray values for each pixel) to obtain a digital chromosome image for the analysis. The G-banding image was processed by the following three steps: correction of lighting fluctuations and illumination distortion to obtain the original image; enhancement of the contrast of the whole chromosome image and also the bands; and smoothing of the enhanced image to obtain the clearer image. This processing was carried out semiautomatically based on the program constructed for the G-banding image, and the processed images were recorded on floppy disks and/or photofilms using a color image recorder. Detailed image processing is presented later demonstrating the digital images obtained.

## Results

Figure 1 shows the typical G-bands of maize chromosomes appearing at the prometaphase to the early metaphase stages. They were obtained by trypsin (Fig. 1 A), SDS (Fig. 1 B), and actinomycin D treatment (Fig. 1 C), respectively. Regardless of the treatments that were employed in the experiment, clear G-bands were demonstrated in all the chromosomes. High resolution G-bands were demonstrated at the prometaphase stage (Fig. 1 A and B) and the banding pattern became coarse toward the early metaphase stage (Fig. 1 C). In the prometaphase chromosomes, the fine G-bands were linked in line in an orderly way along the chromatids, regardless of the treatments adopted. It was also obvious that the bands showed a bead-like configuration, which was observed on both sister chromatids of the chromosome. The fine G-bands fused with each other with the progression of the mitotic stage as shown in Fig. 1 C (early-metaphase), and they almost disappeared or were stained almost uniformly at the mid-metaphase stage. The band-like structures in the mid-metaphase chromosomes appeared mostly as darkly stained blocks rather than bands. The telomeric regions of some chromosomes were hazy, and the bands appeared faint in the prometaphase. The larger knobs that were prominently stained dark became slightly swollen after the G-banding treatment. Two satellites were often observed besides their harboring chromosomes.

Various procedures that have been reported to be effective in the cytology of G-banding were tested. Two-hour pretreatments with either colchicine or colchicine containing ethydium bromide enabled a sufficient number of prometaphase cells to accumulate. They were also effective in keeping the chromosomal structure stable even at the prometaphase, whereas the pretreatments with 2 m*M* 8-hydroxyquinolin and cold water (0°–10 °C) sometimes disturbed the fine structure of the

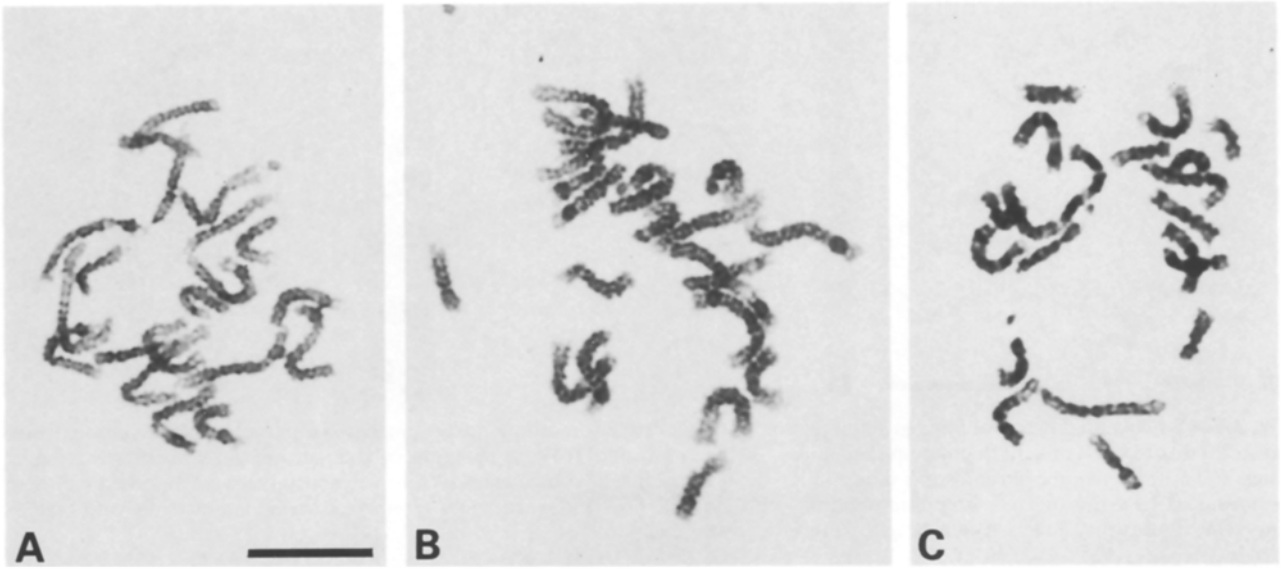


**Fig.1 A–C.** Photomicrographic images of G-banded maize chromosomes. **A** Prometaphase plate treated with trypsin. **B** Prometaphase plate treated with SDS. **C** Early metaphase plate pretreated with actinomycin D. The slides of plates **A** and **B** were prepared by the enzymatic maceration and air-drying method. The slide of plate **C** was prepared by the squashing method. *Bar* indicates 10 μm

prometaphase chromosomes. The hypotonic treatment with Ohnuki's solution (1968) gave clearer bands than without the hypotonic treatment. The hypotonic treatment with 75 m*M* KCl sometimes induced the formation of numerous minute bands randomly arranged along the chromatids. The combination of the enzymatic maceration and air-drying procedures was superior to the squashing method for obtaining the clearer G-bands. Macerozyme R-200 was the most effective when it was combined with Cellulase RS in obtaining good tissue maceration among the pectinases tested under the conditions currently applied.

The chromosomes usually experienced considerable damage and became swollen within several seconds by treatment either with trypsin or SDS. Several procedures, i.e., prolonged drying in a desiccator, baking at 60 °C, post-fixation in fixatives such as methanol-acetic acid (3:1), etc., were examined. Post-fixation with glutaraldehyde gave the best result among them and made the chromosomes sufficiently resistant to the G-banding treatments with trypsin and SDS.

Several reagents that have been known to be effective for the mammal G-banding, i.e., $Na_2HPO_4$, $NaHCO_3$, EDTA-4Na, urea, and Triton X-100, were tested. Although they were also effective in maize G-banding, trypsin and SDS were found to be superior from the viewpoint of both quality and reproducibility of the bands. Wright staining solution was more suitable for the differential staining of the G-bands than the Giemsa solution. When the maize root tips were pretreated with colchicine and actinomycin D, G-bands were also observed without any G-banding treatment by the chemicals listed above, as shown in Fig. 1 C.

Figure 2 shows a sequential representation of the image analysis. The original banding image was obtained after the correction of both lighting fluctuations and illumination distortion by averaging ten identical images and by the shading correction, respectively (Fig. 2 A). Then a digital filter to increase the contrast of the whole image and to enhance the contours of the bands was applied. As a result, the contrast of the boundaries of each band was greatly enhanced, and the faint bands at the telomeric regions became clearly distinguishable (Fig. 2 B). Smoothing of the image was carried out by the 'median' filter (matrix size, $3 \times 3$). After twofold digital enlargement, the centromeric positions of each chromosome were marked by lines, and the mid-rib line of each sister chromatid was traced manually by using a digitizer (Fig. 2 C). The profile lines of the density distribution under the mid-rib lines were printed out. The positions and widths of the bands were used as a reference for the identification of the homologous chromosomes. The length of the long and the short arms of each chromatid was also measured semiautomatically by the CHIAS.

Figure 3 shows the Giemsa-stained and C-banded chromosomes of a metaphase plate (Fig. 3 A and B) and the diagram of the identified chromosomes based on the C-banding pattern and the numerical data (Fig. 3 C). The knobs of pachytene chromosomes correspond to the C-bands of somatic metaphase chromosomes in maize (Aguiar-Perecin and Vosa 1985). C-banding of Oh 43 revealed four pairs of large knobs at *2L* (*L* and *S* repre-

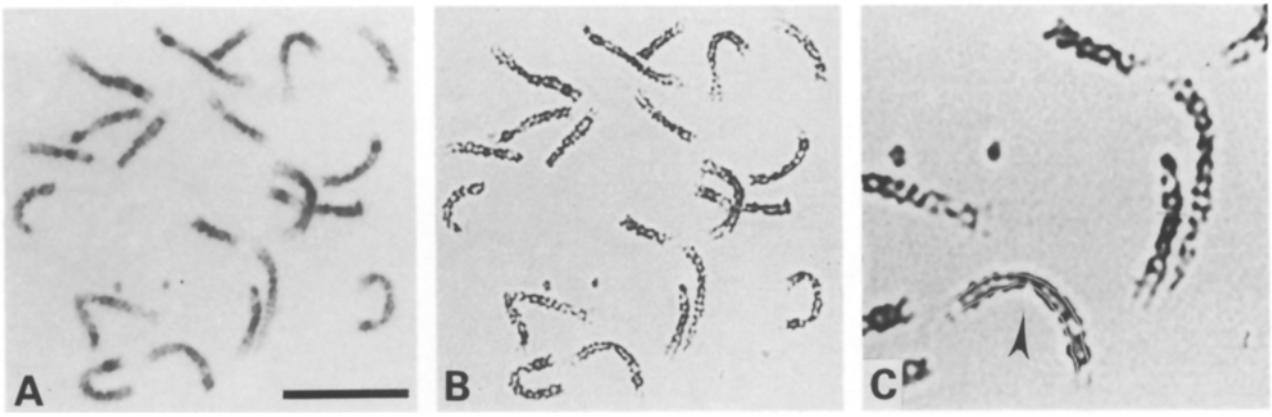


**Fig. 2A–C.** Sequential process of image manipulation of G-banded chromosomes in a prometaphase plate. **A** Original chromosome image after correction of light fluctuations and noises. **B** Enhancement of the contrast of the original image by treatment with a digital filter. **C** Twofold enlarged chromosome image of a part of Fig. 2B. The centromeres of four chromosomes were marked by lines of gray value 0. Mid-ribs of each sister chromatid of a chromosome no. 5 indicated by an *arrow* were traced interactively with overlay lines. *Bar* indicates 10 μm in **A** and **B**, and 5 μm in **C**, respectively

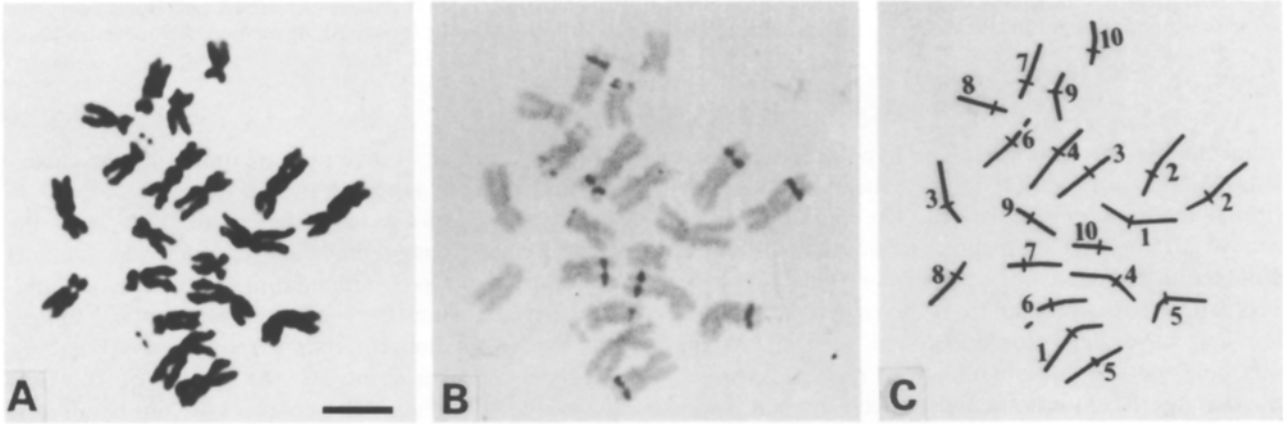


**Fig. 3A–C.** Chromosome identification by the C-banding technique. **A** Giemsa-stained maize chromosomes at metaphase. **B** C-banded maize chromosomes. **C** Chromosome diagram with chromosome number. *Bar* indicates 5 μm

sent the long and the short arm, respectively, i.e., *2L* shows the long arm of maize chromosome 2), *4L, 5L,* and *7L,* a medium size knob at *6L* and a small knob at *9S,* although Chughtai and Steffensen (1987) did not report the presence of knobs at *2L* in this line that they used. All these knobs were always detected, except for the smallest knob at *9S* after the G-banding of the prometaphase chromosomes as prominent bands, and they were useful markers for the identification of the chromosomes after G-banding. The appearance of the smallest knobs at *9S* varied with the plates. The comparison of the numerical data of the metaphase chromosomes identified by the C-banding with the density profile lines of the G-banded prometaphase chromosomes, along with the knob constitution of both the C- and G-banded chromosomes, enabled the chromosome number of the G-banded chromosomes to be completely identified.

Figure 4 shows the karyotype of a G-banded prometaphase plate, arranged in the order of the chromosome number. The G-banding patterns between homologous chromosomes were apparently consistent, although the longer chromosomes, e.g., nos. 1–4, showed some disruptions of the banding patterns between the homologues due to differences in the contraction. Differences in the banding patterns between homologues could be resolved by examining the band fusion and band movement by gyration of the chromatid and/or the chromosome, e.g., chromosomes no. 6 showed an almost consistent banding pattern, assuming that the interstitial two small bands that were on the proximal side of the knob on the long arm of the right homologue fused into one band. The banding pattern of no. 8 chromosomes was quite similar, except for the telomeric faint band on the short arm of the right homologue. Chromosome nos. 5,

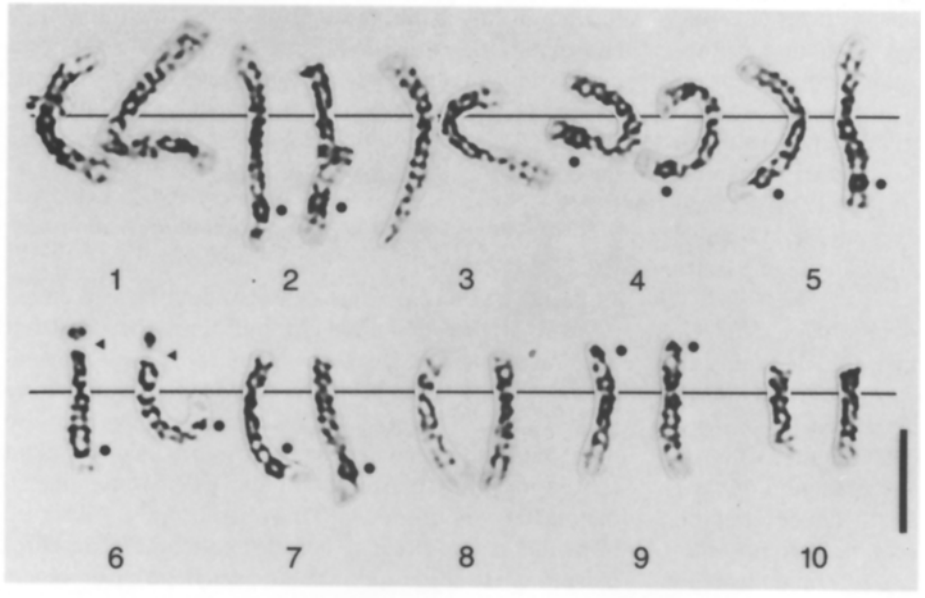


**Fig. 4.** G-banded chromosomes of the plate shown in Fig. 2. The banded chromosomes were arranged in the order of the maize chromosome number. The chromosome images were treated with the digital filter and enlarged twofold on the monitor. The signs (●) and (▸) show the positions of the knobs and NOR regions, respectively. *Bar* indicates 5 μm

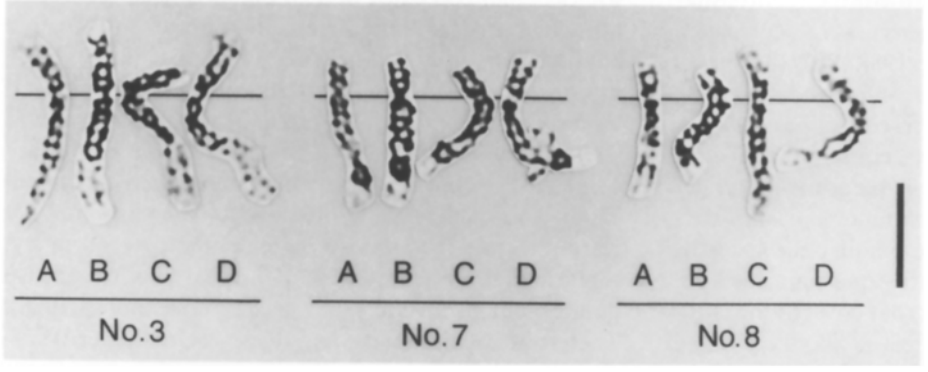


**Fig. 5.** Comparison of the G-banding patterns of the chromosome nos. 3, 7, and 8 obtained from four different plates and by two different methods. *A*, *B*, *C*, and *D* were selected from the plates of Fig. 2, Fig 1A and B, and the plate not shown, respectively. Straight chromosomes with few overlapping regions were selected for the demonstration. *Bar* indicates 5 μm

7, 9, and 10 also showed a similar banding pattern between the homologues.

The variability of the banding patterns was then examined in comparing the G-banded plates obtained by different methods. Figure 5 shows the G-banding pattern of the four chromosomes of chromosome Nos. 3, 7, and 8. Chromosomes in the panel were selected from four different G-banded plates by trypsin treatment (A, C) and SDS treatment (B, D), as they were straight and did not show any overlapping region except for the no. *7D* chromosome. The banding pattern was mostly consistent for these four chromosomes regardless of the G-banding method applied. The differences in the position and width of the bands among them might be due to the fusion of the bands and gyration of the chromosome, etc.

## Discussion

### *Technical aspects of G-banding method in plants*

Many experiments on G-banding have been repeated in plant chromosomes since the G-banding method was developed in mammal chromosomes. The heterochromatic differentiation was reported in *Trillium, Fritillaria, Crepis,* etc., by the experiment of G-banding (Schweizer 1973). G-band-like differentiations were also revealed in the chromosomes of rice (Kurata et al. 1981), red pine (Drewry 1982), and celery (Murata and Orton 1984). Since only Giemsa- or acetocarmine-staining was applied to the prometaphase chromosomes in the case of rice and celery, respectively, the G-band-like differentiation may be due to inherent characteristics of these chromosomes. The trypsin method applied for the banding of the red pine chromosomes resulted in G-bands differing from those of mammal chromosomes both in quality and in the number within a chromosome. Recently, we have observed G-banded chromosomes in *Atriplex patula* (Fukui et al. 1986), barley (Fukui et al. 1989), wheat and rye (Zhu et al. 1986), etc. Chen et al. (1986) and Chen (1988) also reported studies on G-banding in pine and *Lillium*. Another effective G-banding method has been recently reported for the chromosomes of *Vicia hajastata* (Wang and Kao 1988), although the method may not be

applicable under ordinary conditions, as it requires the use of cultured cells as the material, avoidance of pretreatment, and omission of maceration with HCl or an enzymatic mixture. Compared with the methods previously described, the G-banding method demonstrated in this paper enables to obtain G-bands that are characterized by a high resolution and quality, and this method could be applied in many plant species. In fact, G-bands were also recognized in barley, sugar beet, and other crops using this method.

The main procedures required to obtain clear G-bands in plants that would be comparable in quality to those observed in mammals and to apply the technique in many plant species could be summarized as follows. Firstly, the hypotonic treatment with Ohnuki's solution (1968) plays an important role in the induction of clear G-bands. The treatment was reported to induce the spiral or macrocoil structure in human somatic chromosomes (Ohnuki 1968; Rattner and Lin 1985). Although the relationship between G-band and the macrocoil structure of the chromosome has not been clarified, the reports suggested that the two phenomena were correlated (Takayama 1976; Yang and Zhang 1988). It is thus likely that the hypotonic treatment is also effective in inducing the formation of G-bands in the case of maize chromosomes. Secondly, the enzymatic maceration and air-drying procedures were very effective in securing the proper spreading of the chromosomes without cytoplasmic covering. It was sometimes difficult to obtain clear G-bands by the application of the ordinary squashing method, because the cytoplasmic remnants that covered the chromosomes tended to reduce the effect of the trypsin and SDS treatments, and also the staining specificity of the G-bands. Thirdly, the post-fixation with glutaraldehyde was the most important procedure. Glutaraldehyde intercalates the amino acid residues of the chromosomal proteins and enhances the tolerance of the chromosome preparations to the trypsin and SDS treatments. Chromosome preparations subjected to enzymatic maceration usually undergo swelling and degradation changes when they are treated with trypsin or SDS. Long-term drying and post-fixation with methanol-acetic acid did not markedly improve the situation. In contrast, post-fixation with glutaraldehyde enabled a good G-banding pattern to be obtained, as the disruption of the chromosomal matrix could be avoided. Pretreatment of actinomycin D was also effective for G-banding and the effect was also confirmed in barley, wheat, rye, etc. (Zhu et al. 1986).

The image analysis method introduced here was useful in obtaining clearer images of G-banded chromosomes and also in quantifying the G-band positions. The treatment with the contour enhancement digital filter especially contributed to solving problems that arose in the analytical process (Fukui et al. 1989), such as identification of each band, recognition of the orientation of a chromatid mid-rib, crossing position of the sister chromatids, and enhancement of faint bands in the telomeric regions (Fig. 2). The digital filter treatment would also make it easier to quantify the band position by enhancing the contrast of the G-bands.

*Mechanisms of G-banding and chromosome organization in plants*

Although the mechanisms of G-banding have been extensively studied in animal chromosomes, they have not been fully elucidated. It is generally recognized that mitotic G-bands correspond to pachytene chromomeres (Jagiello and Fang 1980). Clear chromomeres are also observed in the pachytene chromosomes of many plant species including maize. Greilhuber (1977) investigated the reason why mitotic G-bands had not been observed in plant chromosomes in contrast to mammalian chromosomes. He suggested that the pachytene chromomere structure is no longer demonstrable in mitotic chromosomes of plants because of their higher degree of contraction compared with the mammalian ones. On the other hand, Anderson et al. (1982) and Bennett et al. (1983) did not observe significant differences in the contraction ratio between the plant and the animal chromosomes. It should be noted that clear G-bands in maize always appeared in the chromosomes with a low degree of condensation and could not be observed in chromosomes toward the mid-metaphase. Stoichiometric error in cytophotometric measurement of nuclear DNA was also pointed out in several plant species (Greilhuber 1988). Therefore, Greilhuber's hypothesis (Greilhuber 1977) could not be ruled out even if some other causes are involved in the lack of appearance of clear G-bands at the metaphase.

Other mechanisms of G-band induction recognized in animal chromosomes include: (i) macrocoiling of a chromosome (Takayama 1976); (ii) AT-rich DNA regions (Schweizer 1981); (iii) late-replicating DNA regions (Holmquist et al. 1982); (iv) alteration and degradation of non-histone chromosomal proteins (Burkholder and Duczek 1982).

As for the first mechanism, the coiled-like structure was found to be correlated with the presence of G-bands in maize chromosomes. It should also be noted, however, that G-bands in plant chromosomes have a bead-like structure, unlike those in animal chromosomes, and become coarse toward the metaphase, as also observed by Wang and Kao (1988). Yang and Zhang (1988) have recently reported on the in situ transition from G-bands to macrocoils by the prolonged trypsin treatment in rye chromosomes, while a transition from the helical structure to the bands was observed in the Chinese hamster chromosomes (Takayama 1976). Regarding the second mechanism, plant chromosomes are differentially stained

in large heterochromatic regions by the DNA base-specific dyes (Schweizer 1976), whereas Q-bands that correspond to the G-bands are revealed in animal chromosomes by an AT-binding dye, quinacrine mustard. This fact suggests that base-specific fine differentiation may not exist in plant chromosomes. Thus, the G-bands observed in maize chromosomes require a reevaluation of the relationship between DNA base-specific sites and G-bands. For the third mechanism, the formation of G-bands can also be ascribed to the differentiation of the DNA replication time within the chromosomes, and it has been shown that late replicating zones correspond to the G-bands in mammals (Holmquist et al. 1982). It was shown, however, that the late replicating DNA existed in interstitial C-bands in *Vicia faba* (Döbel et al. 1978), while they distributed along whole chromosomal regions in *Allium cepa* (Cortes and Escalza 1986). It is thus necessary to examine whether the DNA replicating pattern is related to the G-band structure in plant chromosomes. As for the last mechanism, visible changes in the chromosomes associated with complete removal of histones did not appear in cereal chromosomes (Yang and Zhang 1988). It suggests that the detachment of the certain non-histone chromosomal proteins might be involved for the induction of G-bands also in plant chromosomes. All these results except for the last one suggest that there are some differences in the nature of the G-bands between the plant and the animal chromosomes. Moreover, it is likely that such differences are also present among the chromosomes of different plant species.

Several findings on the nature of the G-bands at the molecular level have been reported recently. 'Housekeeping genes' and 'tissue specific genes', which are replicated at the early and late stages during S phase, respectively, are allocated to the R-band positive regions and G-band positive regions, respectively (Goldman et al. 1984). Specific DNA sequence families correlated to the G-bands can be identified by in situ hybridization (Korenberg and Rykowski 1988).

The G-banding method for plant chromosomes, which has been developed recently, can be applied and practically used in many plant species. Along with the rapid progress of plant biotechnology, a large number of new plant materials are being produced by cell hybridization and gene transfer, which may require the analysis of the chromosomal constitutions or aberrations of these plants. For these purposes the application of the G-banding technique would be of great value. Moreover, the presence of G-bands in plant chromosomes may also pave the way for new areas of research in plant cytology and genetics.

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