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Partial Mitotic Synchronization and Giemsa G-banding in *Allium wakegi*

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Hydroxyurea (HU), a DNA synthesis inhibitor, was tested as synchronizing agent in root-tip meristem of *Allium wakegi*. Roots were treated with 2.5 mM HU for 14 h to accumulate meristem root-tip cells at the G_1/S interface. After release from HU block, the cells re-entered the cell cycle with a high degree of synchrony. Synchronized mitotic frequency of *A. wakegi* was 22.7%, which was about 3.9 times as high as that of the control. The highest metaphase index (23.0%) was obtained when, 6 h after release from the HU block, the roots were treated with 0.05% colchicine for 2 h. Modifying various Giemsa staining protocols defined for animals and a few plant species, G-bands were visualized at prometaphase and metaphase chromosomes of *A. wakegi*. The higher degree of chromosome condensation, the less differential bands could be resolved. This is the first demonstration introduced partial mitotic synchronization into G-banding in plant.

Keywords : *Allium wakegi*, hydroxyurea, partial mitotic synchronization, G-banding

The use of synchronized populations of root-tip or cultured cells has been advantageous for the investigation of events in cell cycle, banding formation, chromosome isolation, and *in situ* hybridization. In the case of higher plants, partial mitotic synchronization was reported in cultured cells or root-meristem tissues after removal of treatments which reduce or block DNA synthesis (Galli and Sala, 1983; Doležel *et al.*, 1992). This can be achieved by addition of DNA synthesis inhibitor such as hydroxyurea, aphidicolin, nalidixic acid and 5-fluorodeoxyuridine, or by changes in the plant growth substance complement of the medium, nutrient starvation, and temporary anaerobic conditions. The meristematic cells can be accumulated at G_1, and their distribution at the G_1/S boundary can be experimentally regulated. However, Constabel *et al.* (1977) have pointed out that besides often long generation tissues, sensitivity of plant cells to changes in media composition and their habit of growing in aggregates are factors limiting the complete success of this approach.

G-bands may be defined as a system of alternating dark and light bands throughout the length of the chromosome. Since G-banding was developed in human chromosome (Sumner *et al.*, 1971), more than a thousand molecular markers and genes have also been mapped on chromosomes based on the fine G-banding patterns in human chromosomes (McAlpine *et al.*, 1987). G-banding pattern is worthy of chromosome identification involving karyotype, chromosome abnormality, origin of cultured cells, gene-mapping and microcytogenetic using high-resolution banding in higher vertebrates. G-banding patterns had been obtained in some higher plants such as *Pinus restinosa* (Drewry, 1982), *Apium graveolens* (Murata and Orton, 1984), *Vicia hajastana* (Wang and Kao, 1988) and *Vicia faba* (Yang and Zhang, 1988). High resolution banding in maize (Kakeda *et al.*, 1990) and G-band-like pattern of *Atriplex patula* (Chen *et al.*, 1986) were also reported. Although being G-banding methods in plants more advanced, its chromosome identification is rather limited than animal. A high resolution and reproducible G-banding methods for plant chromosomes has been required in both basic and applied fields, and many

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studies to obtain G-band were carried out in several laboratories (Chen *et al.*, 1986; Fukui *et al.*, 1986; Zhu *et al.*, 1986). Peffly and de Vries (1993) attempted to compare the Giemsa G-band in the chromosomes of *Allium cepa* L., *A. fistulosum*, and their interspecific F_1 hybrid to detect differences in banding patterns which might serve as cytological markers of introgression and as an aid in karyotyping. But they could only observe the G-bands in prophase chromosomes.

The present paper was focused in the partial mitotic syncronization, followed the Giemsa G-banding on the metaphase chromosomes in *A. wakegi*. Also, the aim of this work was to analyze karyotype and cytological application by using the modified G-banding methods reported previously.

MATERIALS AND METHODS

Cell cycle synchronization

Plants of *Allium wakegi* L. with main roots about 1 cm long were incubated for 14 h in a Hoagland solution containing 2.5 mM hydroxyurea. Then the roots were washed in distilled water thoroughly and immersed in hydroxyurea-free Hoagland solution. Samples of root-tips were taken at 2 h intervals up to 24 h for the analysis of mitotic activity.

Metaphase accumulation

To accumulate mitotic cells at metaphase, root-tips were treated in 0.05% colchicine after 6, 7, or 8 h incubation in hydroxyurea-free Hoagland solution. Root tips were taken 1 or 2 h after the incubation in colchicine for analysis of the frequency of metaphase. Mitotic index and metaphase frequency were analysed on squash preparation. Samples of root-tips were fixed for 3 h in acetic ethanol mixture (1 : 3, v/v). Mitotic index was calculated from 5000 cells and metaphase frequency was obtained from 1000 cells with five repeats.

Giemsa G-banding

Root-tips were pretreated with 0.05% colchicine solution containing 10 ppm ethydium bromide for 2 h after 6 h recovery from Hoagland solution. The material was subsequently immersed in the Ohnuki's hypotonic solution (55 mM KCl, 55 mM $NaNO_3$, 55 mM CH_3COONa, 10 : 5 : 2; Ohnuki, 1968) for 1 h at room temperature, washed thoroughly in distilled water and fixed in acetic methanol (1 : 3, v/v) at 4℃ for 24 h. Fixed root-tips were rinsed in tap water for about 10 min and macerated in an enzymatic mixture (pH 4.2) containing 0.5% cellulase and 0.5% pectinase for 24 h at room temperature in a 1.5 mL Eppendorf tube. After rinsing with distilled water two or three times, each macerated root-tip was cut into small pieces with a sharp-pointed tweezer with addition of fresh fixative. Smashed root-tips were centrifuged at speed of 1000 rpm for 5 min with 2 times. 2-3 drops of suspension were plated on a slide dipped in 50% ethanol, slide heated on alcohol lamp immediately and dried in the air over 2 weeks. The preparations were transferred into 0.01 % trypsin solution dissolved in 0.02% EDTA-CMF at 4-10℃ for 30 s. The slides were rinsed in 95% ethanol, 0.85% NaCl solution, and then were transferred into 5.3 M urea dissolved in the mixture of 2 parts of 0.02% EDTA and 1 part of 1/15 M phosphate buffer (pH 7.4) at room temperature for 1 min. Then, the slides were rinsed in 95% ethanol, and 0.85% NaCl solution again. The slides were stained in 2% Giemsa solution for 8 min, rinsed in tap water and dried in the air. To compare banding patterns with various banding methods, either trypsin or urea alone was employed.

For the comparison of G-banding and C-banding, C-banding using BSG method was employed.

RESULTS

Cell cycle synchronization

Mitotic index decreased slowly as soon as treated with 2.5 mM hydroxyurea in root-tip of *A. wakegi*. No cells undergoing mitosis were found after 14 h hydroxyurea treatment. After a release from the hydroxyurea block, 22.7% of the cells were found to be in mitosis. This value was 3.9 times higher than that of the contol. After then, the values were decreased gradually, and observed second high mitotic peak that was corresponding to about 2.6 times higher than that of the control at 17 h. The span between the first high peak at 8 h and second peak at 17

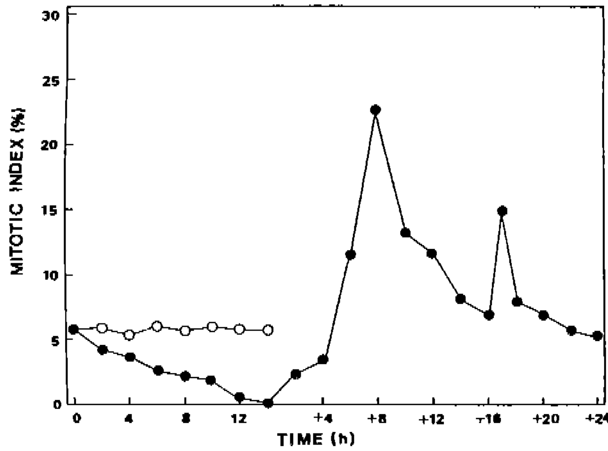

Fig. 1. Variation of the mitotic indices during 2.5 mM hydroxyurea treatment (up to 14 h) and after its removal (up to +24 h) in *A. wakegi*. Empty dots show mitotic indices in control root-tip cells.

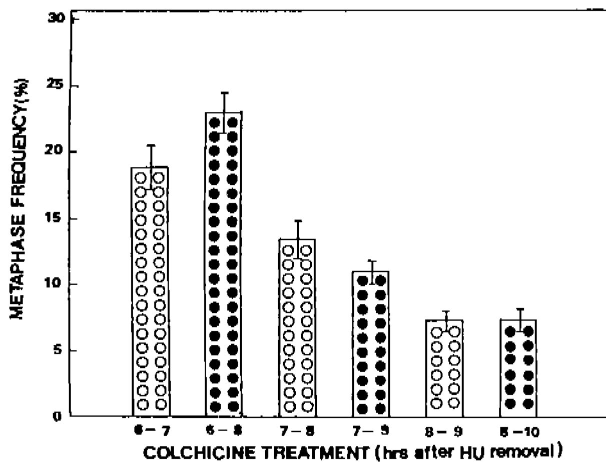

Fig. 2. Variation of the metaphase frequency in *A. wakegi* root-tips (Mean± SE) treated with 0.05% colchicine for various periods during recovery from the HU block. Empty dots were represented for 1 h and filled dots were represented for 2 h pretreatment.

h in mitotic indices means to be cell cycle of 9 h in this species (Fig. 1).

Metaphase frequency analysis

The highest metaphase index (23%) was achieved when, 6 h after release from the hydroxyurea block, the roots were exposed to colchicine for 2 h. Standard error is not exceeded 2% in five repeats (Fig. 2).

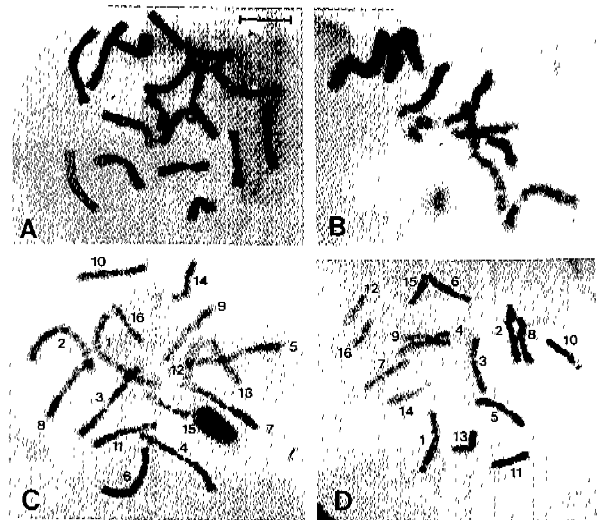

Fig. 3. Photomicrographs images of G-banded chromosomes in *A. wakegi*. A, Metaphase plate treated with trypsin; B, Metaphase plate treated with urea; C and D, Prometaphase and metaphase plate treated with trypsin-urea. Bar indicates 10 μm.

Giemsa G-banding

G-banding patterns were obtained by various treatments (Fig. 3). In banding pattern by trypsin, structural bands were not visible, but were characterized the empty appearance in each end of chromosomes (Fig. 3A). The chromosomes treated with urea were visualized diffused many bands along entire chromosome than trypsin treated chromosomes, but outlines in chromosomes edges were obscured (Fig. 3B). In this study, clear G-banding patterns were obtained with trypsin-urea double treatment methods. This result seems to be complemented the defects which occurred in each treatment. The higher degree of chromosome condensation, the less differential bands could be resolved (Fig. 3C, D).

Giemsa C-banding and G-banding patterns were compared (Fig. 4). C-banding patterns of *A. wakegi* was more distintive in the ends of both arms of all chromosomes, centromeric region, satellite region of chromosome 14 and some interstitial regions of both arms in chromosomes 3, 7, 15, and 16. In G-banding patterns, many bands were observed along the entire length of metaphase chromosomes. The result of G-banding treatment pattern is contrasted to C-band pattern in satellite region of chromosome 14 especia-

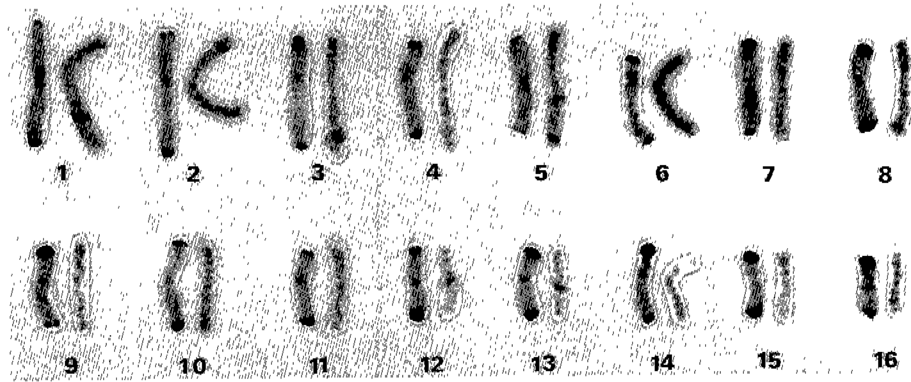

Fig. 4. Karyotypes of C-banded (left) and G-banded (right) metaphase chromosomes in *A. wakegi*.

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DISCUSSION

G-banding of plant chromosome is nearly successful despite of many cytogenetist's efforts. There are reasons of these failures in plants owing to chracteristic cell wall, relatively high condensation of metaphase chromosome than animals involving human and low frequency of metaphase cells after spirit-flame drying technique. The prerequisite for preparation of G-banding is to arrest many metaphase. The authors obtained the highest mitotic index of 22.7% with partial mitotic synchronization using DNA synthesis inhibitor which is correspond to 3.9 times higher than that of the control (Fig. 1). This is lower frequency than that of Doležel *et al.* (1992) obtained in mitotic synchronization induction in *Vicia faba* root-tips, but higher than that of conventional preparation methods using spindle-formation inhibitor alone. Conia *et al.* (1987) took advantage of spontaneously occurring synchrony of the first division of the cultured leaf protoplasts of *Petunia hybrida* and *Nicotiana plumbaginifolia*, and combined it with a colchicine treatment to accumulate high frequency of metaphase cells. Also, Hadlaczky *et al.* (1983) reported exposing cell suspension to 0.1% colchicine without hydroxyurea treatment, accumulates a sufficiently high frequency of metaphase cells in *Papaver sominiferum*. Unlike other authors (Hadlaczky *et al.*, 1983; Conia *et al.*, 1987; Arumuganathan *et al.*, 1991) and similarly to Mii *et al.* (1987), the authors have achieved a very high frequency of metaphase cells by a combined treatment with a DNA synthesis inhibitor and a mitotic spindle inhibitor (Fig. 2). The advantage of the combined protocol is that accumulation of sufficient numbers of cells in metaphase can be achieved after a relatively short treatment with a mitotic poison. This avoids the chromosome decondensation and micronuclei formation usually observed after longer colchicine treatments (Conia *et al.*, 1987; Arumuganathan *et al.*, 1991). The mitotic index showed a first peak 8 h and a second peak at time 17 h (Fig. 1). From this result, it is likely that cell cycle of this species is 9 h. Galli and Sala (1983) reported the cell cycle of *Haploppapus gracilis* root-tip cells is 11 h based on the result using DNA synthesis inhibitor, aphidicolin.

G-banding patterns comparable to those in higher vertebrates, however, have not been demonstrated in plant chromosomes for more than 20 years despite the efforts of plant cytologists. 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). Although Anderson *et al.* (1982) have concluded that there are no consistent differences in the degree of compaction between plant and animal chromosomes, this study obtained the corresponding results with Greilhuber's hypothesis (Fig. 3C, D).

Since Drewry (1982) reported G-banding in *Pinus restinosa* using trypsin-Giemsa methods, G-banding in a few plant species has been reported. The mechanisms of the G-band formation or its fields are still not know well. The authors attempted to improve the methods from various G-banding methods

which reported previously in animals or plants. First, G-banding method using trypsin was developed by Sumner *et al.* (1971) in animal chromosomes. Trypsin banding was claimed to be less sensitive to the age of the slide, even slides up to three years old being usable (Seabright, 1971). Trypsin tends to produce a fuzzy outline to the chromosomes, but chromosome outlines was clearly appeared in the present study (Fig. 3A). Second, urea known as effect of protein extraction from chromatin was very effective in G-banding of animal chromosomes (Berger, 1972; Kato and Yosida, 1972; Shiraishi and Yosida, 1972), but tends to produce a fuzzy outline to the chromosomes with many bands (Fig. 3B). Band patterns of high resolution were obtained using trypsin-urea double treatments (Fig. 3C). Peffley and de Vries (1993) reported that G-banding appeared in the chromosomes of *Allium cepa, A. fistulosum,* and their interspecific hybrid (*A. fistulosum* × *A. cepa*) using $NaHCO_3$ method suggested by Wang and Kao (1988), and trypsin method suggested by Drewry (1982). Their experimental procedures were omitted step of alkali treatment in preparation. G-banding of their result was visualized in prophase, not in metaphase chromosomes. In contrast to their reports, distinctive G-banding was observed at prometaphase or metaphase of *A. wakegi* in this paper. The banding patterns obtained resembles the dotted banding patterns along the entire chromosome complement which have been reported by Drewry (1982), Murata and Orton (1984), Wang and Kao (1988), and Yang and Zhang (1988) in several plant species. In *A. wakegi*, the number of bands visible along the entire length of chromosomes in metaphase was reduced, compared to that shown in prometaphase chromosomes (Fig. 3C, D). Human chromosomes also show a reduction of G-band resolution with increasing chromosome condensation (Bickmore and Sumner, 1989). Unlike G-bands, the number of C-bands does not depend upon the rate of chromosome condensation. C-bands were marked in constitutive heterochromatin selectively, while G-bands with various sizes and gaps were distributed on entire chromosome length (Fig. 4). For elucidating the mechanisms of G-banding, more detailed research will have to be done in the future.

In conclusion, partial mitotic synchronization using hydroxyurea is useful for plant chromosome isolation, *in situ* hybridization, chromosome band formation, and so on. G-banding pattern of this study seems to be important for identification of the genomic organization of *A. wakegi.* Tashiro (1980, 1981, 1984) suggested that *A. wakegi* originated from *A. fistulosum* and *A. ascalonicum* in the basis of interspecific cross, fertility, meiotic configuration, and genomic analysis. It is likely that the futher analysis of G-banding patterns from these species will confirm Tashiro's results.

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쪽파의 部分的 有絲分裂의 同期性 誘導와 Giemsa G-分染

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적 요

Hydroxyurea(HU)는 식물의 근단 분열 조직에서 부분적 세포 분열의 동기성을 유도하는 DNA 합성 저해제이다. 쪽파의 근단 조직을 2.5 mM HU에 처리한지 14시간만에 G_1기와 S기 사이에 세포들이 모이도록 유도할 수 있었으며 이후 HU를 제거하고 다시 세포 분열을 진행시킨 결과, 세포 분열이 다시 회복되면서 8시간째 가장 높은 세포 분열 지수인 22.7%가 관찰되었다. 이 지수는 대조군의 평균 세포 분열 지수인 5.8%의 약 3.9배에 상당하는 지수이다. 이후 17시간째 다시 한 번 높은 세포 분열 지수가 관찰됨으로써 이 종의 세포 주기가 9시간임을 알 수 있었다. 많은 중기 세포를 얻기 위해 0.05% colchicine으로 전처리한 결과 세포 분열을 회복시킨지 6시간째 2시간 전처리한 경우에서 가장 높은 빈도의 중기 세포(23%)를 관찰할 수 있었다. 동 · 식물의 G-분염에 효과가 있는 여러 가지 방법을 수정하여 쪽파에서 G-분염을 실시한 결과 전중기상과 중기상에서 뚜렷한 G-분염상을 관찰할 수 있었다. 전중기상과 중기상을 비교한 결과, 염색체 응축에 따라 G-분염의 밴드 수는 감소하였다. 본 연구는 식물에서는 처음으로 세포 분열의 동기성을 G-분염에 도입한 것이다.

주요어 : 쪽파, hydroxyurea, 부분적 유사 분열의 동기성, G-분염

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