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Partial Synchronization of Cell Division and Micronucleation in Suspension-cultured Cells of *Hemerocallis hybrida*: The effects of Hydroxyurea and Various Spindle Toxins

Hiroyuki Saito and Masaru Nakano*

Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Niigata, Niigata 950-2181, Japan

For the production of intergeneric hybrid plants with partial alien genome via microprotoplast fusion in the Liliaceous ornamentals, the effects of the DNA synthesis inhibitor, hydroxyurea (HU), and the spindle toxins, colchicine (COL), oryzalin (ORY), amiprophos-methyl (APM), butamiphos (BUT), isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) and propyzamide (PRO) on the metaphase index (MI) and the percentage of micronucleated cells (micronucleus index; MNI) were examined in cell suspension cultures of *Hemerocallis hybrida* cv. Stella d'Oro. Suspension cells were subcultured every three days in MS medium containing 10 mg l^{-1} picloram. Although MI was only 2-3 % in the asynchronous control cultures, it increased up to 8.9 % and 9.7 % by treatment of the cultures with HU and COL, respectively. In addition, MI was further increased by using the sequential treatments of the cultures with HU and each spindle toxin: the highest MI of 30.5 % was obtained by treatment with 2 mM HU for 24 h followed by that with 250 μM COL for 20 h. COL and ORY were more effective for synchronizing cell division than the other four spindle toxins. The effects of various spindle toxin treatments on the micronucleation of suspension cultures were also examined by combining the HU pre-treatment for 24 h. Among the six spindle toxins, COL and ORY induced few micronuclei, whereas APM, BUT, CIPC and PRO induced micronucleation in cells to various extents. The most effective treatment for micronucleation was that with 8 μM PRO for 66 h, where MNI was 14.7 % and the number of micronuclei per cell ranged from 1-7 were obtained.

Key Words: ***Hemerocallis hybrida*, DNA synthesis inhibitor, micronucleation, metaphase index (MI), spindle toxin.**

Introduction

Somatic hybridization via protoplast fusion can be a useful approach for transferring polygenically-controlled traits, unidentified and uncloned genes between sexually incompatible species. Since most of the hybrids obtained via symmetric protoplast fusion may contain numerous undesired genes, however, repeated backcrossing and selection are required for eliminating the undesirable traits. Furthermore, the obtained hybrids often show chromosome loss, weakness or sterility probably due to somatic incompatibility (Harms 1983). To solve these problems, asymmetric hybridization has been carried out using irradiated protoplasts (Waara and Glimelius 1995). However, irradiation treatments often result in chromosome breakage and deletion at random (Negrutiu *et al.* 1989, Wijbrandi *et al.* 1990). Recently, an asymmetric hybridization technique using microprotoplasts has been established in the Solanaceous species. Since microprotoplasts contain only one or a few intact chromosomes, chromosome addition lines with the alien chromosomes can be produced in a single step (Ramulu *et al.* 1993, 1995, 1996a, b).

We aim to produce intergeneric hybrid plants with only one or a few alien chromosomes via microprotoplast fusion for genetic improvement and chromosome studies in the Liliaceous ornamentals. For applying this technique, it is prerequisite to establish an efficient system for mass-preparation of micronucleated cells through synchronized cell division (Ramulu *et al.* 1988a, b, 1994, Verhoeven *et al.* 1990, Verhoeven and Ramulu 1991). Highly-synchronized cell populations have generally been obtained by treating fast-growing cell suspension cultures with DNA synthesis inhibitors and/or spindle toxins (Assaf *et al.* 1984, Szabados *et al.* 1981, Pan *et al.* 1993). Therefore, in the present study, we examined as a first step toward asymmetric hybridization via microprotoplast fusion the effects of the DNA synthesis inhibitor hydroxyurea (HU) and various spindle toxins on the synchronization of cell division and micronucleation in cell suspension cultures of *Hemerocallis hybrida*, a Liliaceous ornamental plant.

Materials and Methods

Plant material and establishment of cell suspension cultures

Hemerocallis hybrida cv. Stella d'Oro (2n = 2x = 22) was used in the present study. For callus induction, root segments isolated from *in vitro*-grown plantlets were cultured for two months on MS (Murashige and Skoog 1962) medium containing 10 mg l^{-1} picloram, 3% (w/v) sucrose and 0.2% (w/v) gellan gum at 25°C in the dark. For inducing cell suspension cultures, root-derived calli were transferred into the same medium but without gellan gum, and cultured

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*Corresponding author (e-mail: mnakano@agr.niigata-u.ac.jp)

at 25°C in the dark on a rotary shaker (100 cycle min^{-1}). About one month after transfer into the liquid medium, vigorously growing cell suspension cultures were established. Suspension cells were subcultured every three days at a density of 1 ml packed cell volume (PCV) per 30 ml of the fresh medium. The PCV of subcultured cells stability increased to about 2.5-fold in three days.

Treatments with HU and various spindle toxins

Hydroxyurea (HU) was obtained from Sigma Chemical Co., St. Louis, Mo., USA. A stock solution of HU was prepared at 0.6 M in water-free dimethylsulfoxide (DMSO). Spindle toxins: colchicine (COL) was purchased from Kanto Chemical Ltd., Tokyo, Japan; oryzalin {ORY, 3,5-dinitro-4-(dipropylamino)-benzolsulfonamid} from Riedle-de Haën AG, Seelze, Germany; amiprophos-methyl {APM, *O*-methyl-*O*-*O*-(4-methyl-6-nitrophenyl)-*N*-isopropyl-phosphorothioamidate} from Hayashi Pure Chemical Industries Ltd., Osaka, Japan; butamiphos {BUT, *O*-ethyl-*O*-(5-methyl-2-nitrophenyl)*N*-(1-methylpropyl) phosphoramidothioate} and propyzamide {PRO, 3,5-dichloro-*N*-(1,1-dimethylpropyl) benzamide} from Wako Pure Chemical Industries Ltd., Osaka, Japan; and CIPC {isopropyl *N*-(3-chlorophenyl) carbamate} from Sigma Chemical Co., St. Louis, Mo., USA. A stock solution of COL (1.9 mM) was prepared in water and filter-sterilized. Stock solutions of ORY (0.6 mM), APM (0.96 mM), BUT (3 mM), PRO (0.6 mM) and CIPC (1.1 mM) were prepared in DMSO. All stock solutions were stored at −20°C. HU at final concentrations of 1 to 4 mM was added to cell suspension cultures 12 h after subculture. After treatment with HU for 12 or 24 h, cells were washed three times with HU-free fresh medium and then re-cultured in a medium containing each spindle toxin. Cultures were maintained at 25°C in the dark on a rotary shaker (100 cycle min^{-1}).

Cytology

The suspension cells (0.5-1 ml PCV) were randomly sampled at certain intervals after subculture. Samples were fixed in ethanol:acetic acid (3:1, v/v) at 0°C for over 24 h. After hydrolyzing with 1 N HCl:45% acetic acid (1:2, v/v) at 60°C for 10-15 s, samples were stained with 1% aceto-orcein and squashed under a cover glass. For determining MI (number of cells at metaphase/total number of cells observed × 100%) and MNI (number of micronucleated cells/total number of cells observed × 100%), at least 2,000 and 500 cells per experiment, respectively, were observed. Value in Figs. 1-4 and 6 represent the mean of two independent experiments.

Results

Synchronization of cell division

In the control cultures without any synchronization treatments, MI was constantly 2-3% during the 3-day culture period (Figs. 1-4 and 5B). For examining the effect on MI of HU, suspension cells were treated with 1 mM HU. Figure 1 shows the effect of HU treatment for 12 or 24 h on MI. Although MI decreased during HU treatment, it gradually increased after the release from HU inhibition. HU treatment for 24 h was more effective for increasing MI than that for 12 h. The maximum MI was obtained 12 h after the release from HU treatment for 24 h and reached up to 8.9% (Fig. 1). The effect of COL on MI was also examined by treatment only with 125 μM COL. MI increased soon after the addition of COL into the culture medium. The peak of MI (9.7%) appeared 6 h after the initiation of COL treatment, thereafter MI decreased (Fig. 2).

MI was further increased by combining HU and COL treatments. The concentration of HU during the pre-treatment greatly affected MI during the COL treatment, and the maximum MI of 27.9% was obtained by the sequential treatments initially with 2 mM HU for 24 h and then with 125 μM COL for 20 h (Fig. 3). Therefore, in the following experiments, all treatments with spindle toxins were combined with the pre-treatment with 2 mM HU for 24 h.

Figures 4 and 5 show the effects of six spindle toxins on MI following the pre-treatment with HU. COL and ORY were more effective for increasing MI than the other chemicals. With COL treatment, the maximum MI was obtained after 20 h at all concentrations tested. Among different con-

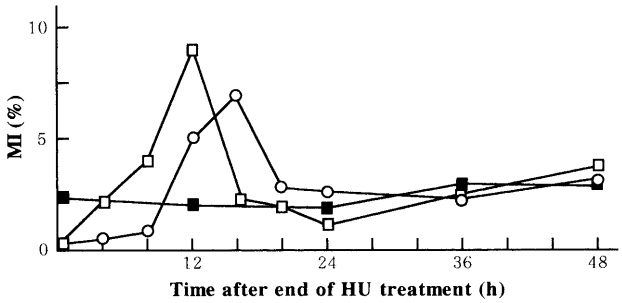

Fig. 1. Change of MI in suspension cultures with the lapse of time after 1 mM HU treatments for 12 or 24 h. HU was added 12 h after subculturing. ○, 12 h; □, 24 h; ■, without HU treatment (control).

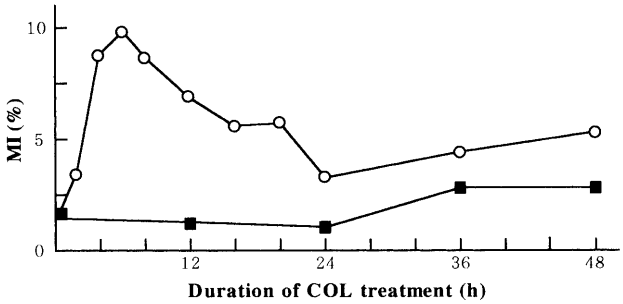

Fig. 2. Change of MI in suspension cultures with the lapse of time after the addition of COL. COL was added 36 h after subculturing. ○, 125 μM COL; ■, without COL treatment (control).

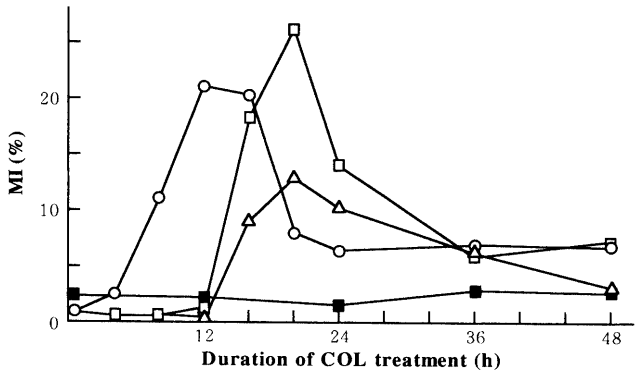

Fig. 3. Change of MI with the lapse of time after the addition of 125 μM COL in suspension cultures pre-treated with various concentrations of HU for 24 h. HU: ○, 1 mM; □, 2 mM; △, 4 mM. ■, without both HU and COL treatments (control).

centrations of COL, 250 μM gave the highest MI of 30.5% (Figs. 4A and 5A). In ORY treatments, the highest MI of 24.5% was obtained 20 h after the addition of 5 μM ORY (Fig. 4A). In most cells arrested in metaphase after either COL or ORY treatment, chromosomes gathered into a single group in the cell. APM, BUT, CIPC and PRO were less effective for increasing MI. The maximum MI for each treatment was obtained 16-24 h after the initiation of treatment: 12.3% with 16 μM APM, 17.1% with 5 μM BUT, 7.6% with 5 μM CIPC and 7.9% with 8 μM PRO (Figs. 4B, C).

Micronucleation

The effects of different spindle toxins on the micronucleation of suspension cells were examined by using the cultures pre-treated with 2 mM HU for 24 h. MNI was strikingly different according to the kind of spindle toxins. Although both COL and ORY treatments induced a relatively high MI, few micronucleated cells were obtained with these treatments (data not shown). On the other hand, APM, BUT, CIPC and PRO induced micronucleation to various extents. Figure 6 shows the change in MNI with the lapse of time after the addition of APM, BUT, CIPC or PRO treatments. Micronuclei started to be observed about 12 h after the onset of each treatment, and then the number of micronucleated cells gradually increased thereafter. In the treatments with APM, BUT and CIPC, the maximum MNI was obtained after 48, 36 and 60 h, respectively, but these were only 3.2-4.7% (Fig. 6A-C). On the other hand, PRO drastically increased MNI, and the maximum MNI (14.7%) was obtained after 66 h of treatment with 8 μM PRO (Fig. 6D).

The number of micronuclei per micronucleated cell was also different according to the kind of spindle toxins. Most of the micronucleated cells obtained with APM, BUT and CIPC had only 1-3 micronuclei per cell. On the other hand, PRO treatments induced a higher yield of micronuclei, ranging from 1 to 7 per cell (Figs. 7 and 8).

Discussion

Our results showed that the sequential treatments with HU and COL were effective for synchronizing cell division of suspension-cultured cells of *Hemerocallis hybrida* cv. Stella d'Oro. The highest MI (30.5%) obtained with these treatments was about ten-fold as compared to MI in the control asynchronous cultures. In most of the previous studies on the synchronization of suspension cultures, the highest MI was 10-35% (Eriksson 1966, Ramulu *et al.* 1988a,b, 1991, 1994), although a much higher MI (60-90%) was obtained for *Pestroselinum hortence* (Szabados *et al.* 1981), *Nicotiana plumbaginifolia* (Ramulu *et al.* 1994) and tobacco BY-2 cell line (Nagata and Kumagai 1999). In monocotyledonous species, only one paper has previously appeared on the synchronization of suspension cultures, in which a relatively high MI (32%) was obtained for *Triticum monococcum* (Szabados *et al.* 1981). To our knowledge, the present study is the first to demonstrate the synchronization of cell division using suspension cultures in Liliaceous species. In addition, the highest MI obtained here was comparable to those reported previously for other species. Since the DNA synthesis inhibitor, aphidicolin, was reported to show a high synchronization effect in suspension cultures of *Petunia hybrida* (Assaf *et al.* 1984), it should be determined whether the other DNA synthesis toxins show stimulatory effects higher than that of HU on the synchronization in *Hemerocallis*.

In the present study, both synchronization and micronucleation were greatly affected by the kind of spindle toxins. Among six chemicals, COL and ORY induced a relatively high MI, but few micronucleated cells were obtained with them. On the other hand, APM, BUT, CIPC and PRO could induce micronucleation to various extents, although these chemicals were less effective for increasing MI. Spindle toxins inhibit microtubule polymerization and thus normal centromeric division. Therefore, cell-cycle progression is prevented in metaphase. COL, which belongs to plant alkaloids, has been reported to bind with a low affinity to tubulins in plant cells unlike in mammals (Dustin 1984, Morejohn *et al.* 1987b). On the other hand, the dinitroaniline herbicide, ORY, and the phospholic amide herbicide, APM and BUT, as compared to COL, have been shown to be effective for inhibiting polymerizing microtubules and thus accumulating metaphase cells (Morejhon *et al.* 1987a, Verhoeven *et al.* 1990, Ramulu *et al.* 1991, 1994). Furthermore, these three chemicals effectively induced micronucleation in suspension-cultured cells of *Nicotiana plumbaginifolia* and *Solanum tuberosum* (Verhoeven *et al.* 1991, Ramulu *et al.* 1994). In the present study, however, COL was more effective for inducing high MI in suspension cultures of *Hemerocallis* than the other spindle toxins including APM and BUT. In addition, both APM and BUT had rather small effects on the induction of micronucleation. The disagreement in the effects of spindle toxins between this and previous (Verhoeven *et al.* 1990, Ramulu *et al.*

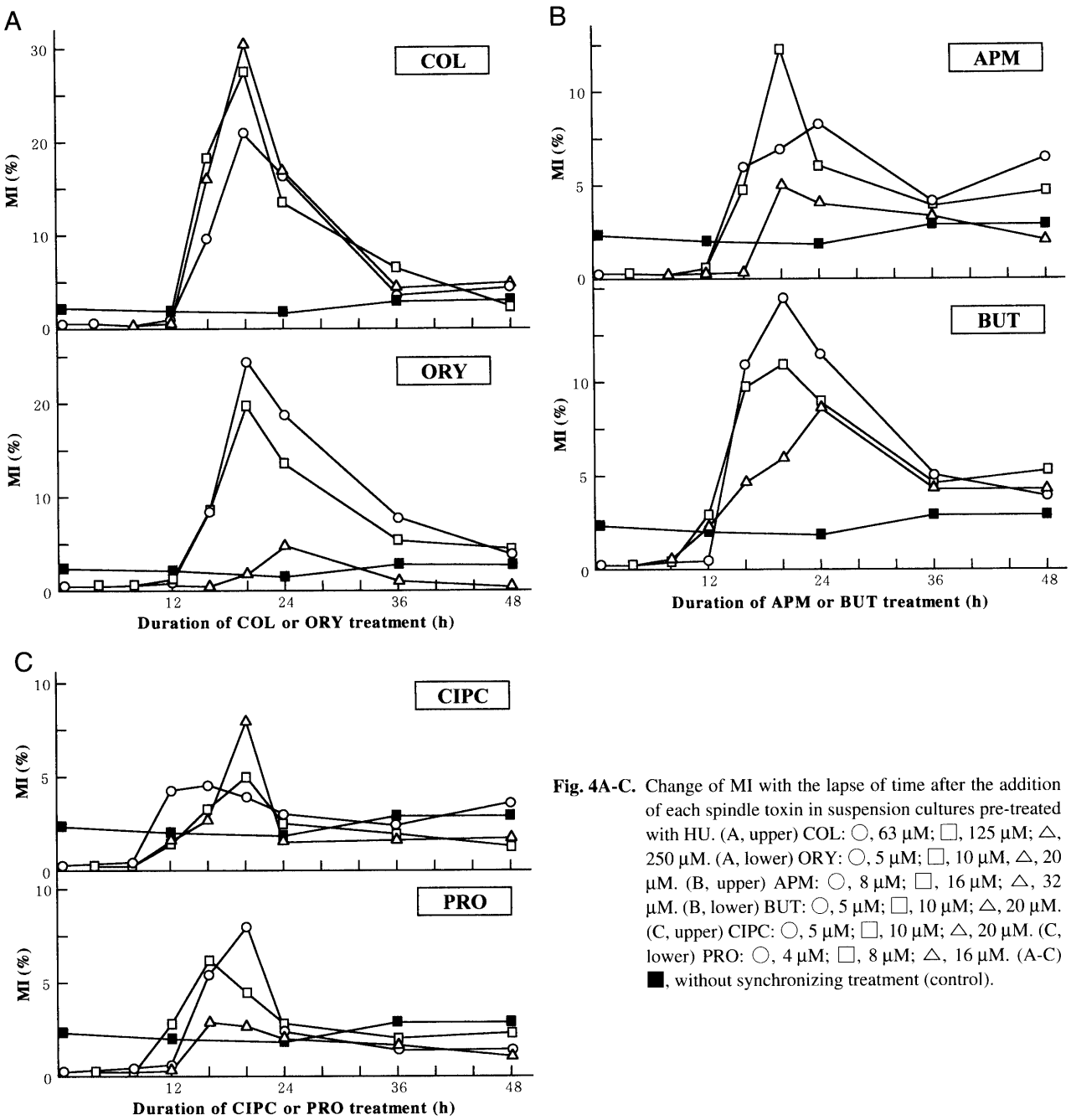

Fig. 4A-C. Change of MI with the lapse of time after the addition of each spindle toxin in suspension cultures pre-treated with HU. (A, upper) COL: ○, 63 μM; □, 125 μM; △, 250 μM. (A, lower) ORY: ○, 5 μM; □, 10 μM, △, 20 μM. (B, upper) APM: ○, 8 μM; □, 16 μM; △, 32 μM. (B, lower) BUT: ○, 5 μM; □, 10 μM; △, 20 μM. (C, upper) CIPC: ○, 5 μM; □, 10 μM; △, 20 μM. (C, lower) PRO: ○, 4 μM; □, 8 μM; △, 16 μM. (A-C) ■, without synchronizing treatment (control).

1991, 1994) studies may be due to the differences in the number and size of chromosomes, division activity of cells and/or other physiological conditions of cells.

For efficient micronucleation, it is also necessary to induce cells with scattering chromosomes throughout the cytoplasm at metaphase (Verhoeven *et al.* 1990, Ramulu *et al.* 1991, 1994). Actions of COL in most plant species have been known to mainly induce the C-mitosis (Levan 1954), which is called ball-metaphase (cell with clumped chromosomes, Deysson 1968, Verhoeven *et al.* 1990). On the other hand, ORY, APM and BUT increased the frequency of scattered-metaphase (cell with scattered chromosomes, Verhoeven *et al.* 1990), which resulted in increasing MNI and the number of micronuclei per cell in suspension cultures of *Nicotiana plumbaginifolia* and *Solanum tuberosum* (Ramulu *et al.* 1991, 1994). In the present study, since ball-metaphase was frequently observed in ORY- as well as COL-treated suspension cultures of *Hemerocallis*, low frequencies of micronucleation induced by both chemicals may be due to less scattering of chromosomes.

Regulation of the transition from metaphase to telophase in the cell-cycle (telophasing), which is one of the actions of spindle toxins, may also be an important factor for increasing MI and MNI (Matsui *et al.* 1982). Several studies

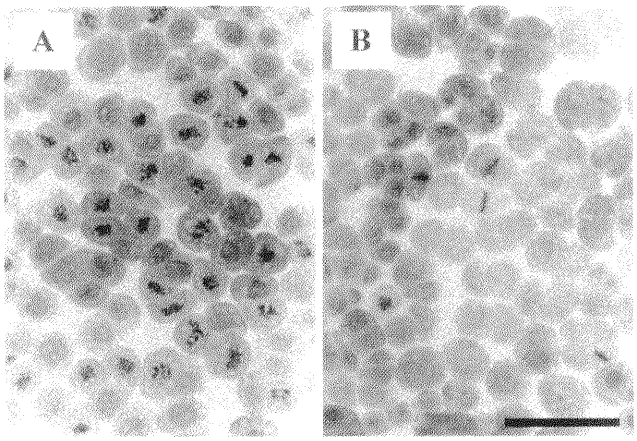

Fig. 5A, B. Microphotographs showing the suspension-cultured cells after the sequential treatments with 2 mM HU for 24 h and then with 250 μM COL for 20 h (A) or without synchronizing treatment (B). Bars = 100 μm.

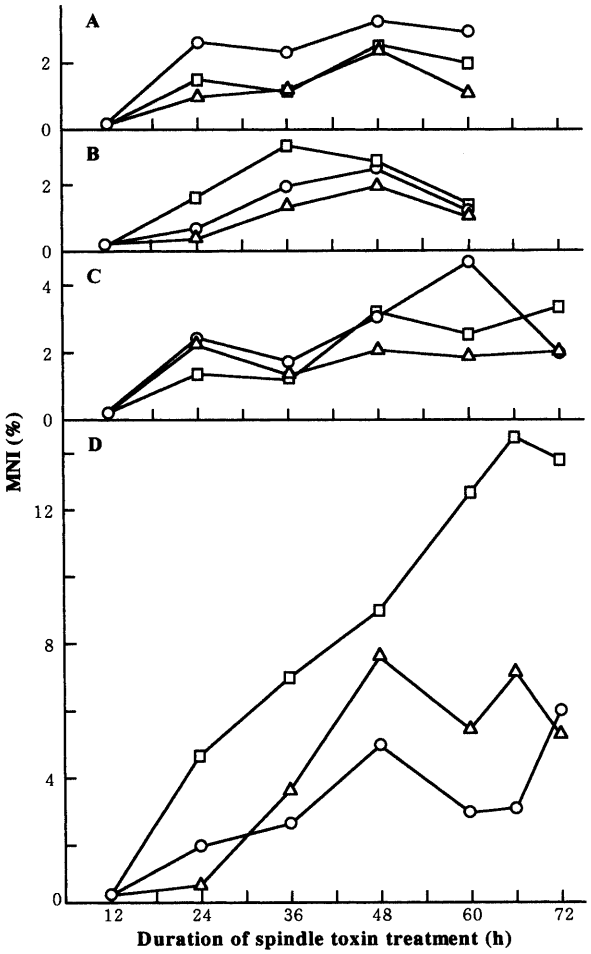

Fig. 6A-D. Change of MNI after the addition of each spindle toxin in suspension cultures pre-treated with HU. (A) APM: ○, 8 μM; □, 16 μM; △, 32 μM. (B) BUT: ○, 5 μM; □, 10 μM; △, 20 μM. (C) CIPC: ○, 5 μM; □, 10 μM; △, 20 μM. (D) PRO: ○, 4 μM; □, 8 μM; △, 16 μM.

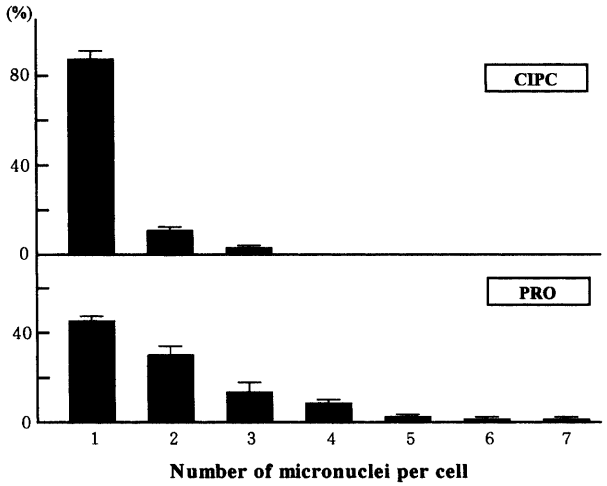

Fig. 7. Frequency distribution of the number of micronuclei per micronucleated cell after the sequential treatments with HU and then with 5 μM CIPC for 60 h (upper) or 8 μM PRO for 66 h (lower). Vertical bars represent the standard error of the mean of four experiments.

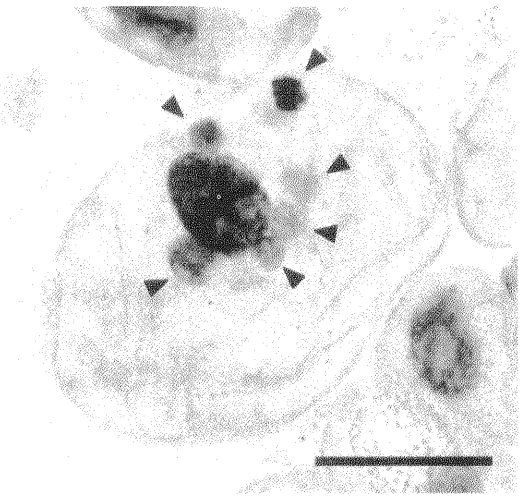

Fig. 8. Microphotograph showing a micronucleated cell with several micronuclei induced after sequential treatments with 2 mM HU for 24 h and then with 8 μM PRO for 66 h. Arrowheads represent micronuclei. Bar = 10 μm.

using both plant and mammalian cells have emphasized the importance of intracellular Ca^{2+} in cell-cycle progression (Hepler 1980, Matsui *et al.* 1982, Wolniak *et al.* 1983, Wolniak and Bart 1985). Matsui *et al.* (1982) indicated that lowering of the cytoplasmic Ca^{2+} level stimulated the reformation of nuclear membrane, and furthermore, a two- to three-fold increase in MNI was accomplished by promoting the reformation through treatment with the Ca^{2+} cheletor, EGTA. Hertel *et al.* (1980) reported that APM and ORY, but not COL, deregulated cytoplasmic Ca^{2+} level and inhibited Ca^{2+} uptake by mitochondria in *Cucurbita pepo* and *Zea mays*. In the present study, some spindle toxins other than

COL might also lower the level of cytoplasmic Ca^{2+} and thus stimulate the reformation of nuclear membranes in suspension cells of *Hemerocallis*, which may lead to the COL-induced high MI, and also lead to APM-, BUT-, CIPC- and PRO-induced micronucleation.

To our knowledge, successful application of the PRO treatment for the induction of micronucleation has not been reported previously in either plant or mammalian cells. PRO increased not only MNI but also the number of micronuclei per cell in suspension cultures of *Hemerocallis*. PRO, which belongs to a class of benzamide herbicides, shows a herbicidal action based on the inhibition of cell division in root-meristematic tissues, and is toxic to many annual and perennial species of the grass family and annual species in the other families (Crovetti and Horron 1970, Roberts and Ricketts 1973). PRO has recently been utilized for synchronizing cell division in cytoskeleton studies using tobacco BY-2 cells (Akashi *et al.* 1988, Kakimoto and Shibaoka 1988, Shibaoka *et al.* 1996). Therefore, PRO may be applicable to a wide range of plant species for both synchronizing cell division and micronucleation. PRO treatments of suspension-cultured cells of *Hemerocallis* are useful for efficient induction of micronucleation, and thus this system can be applied to the partial genome transfer using microprotoplasts in the Liliaceous ornamental plants.

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