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Flow Cytometric Analysis of Partially Synchronized Suspension Cultures of *Hemerocallis hybrida*

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Abstract

Cell division of suspension cultures of *Hemerocallis hybrida* was partially synchronized by treating with hydroxyurea (HU), a DNA synthesis inhibitor, followed by treatment with colchicine (COL), a spindle toxin, as the first step toward an efficient preparation of microprotoplasts. During COL treatment, suspension cells were analyzed for relative DNA contents using flow cytometry (FCM) as well as mitotic index (MI) under microscopy. MI reached a peak after 20 h of COL treatment. In FCM analyses, histogram of asynchronous cultures showed three peaks (2C, 4C and 8C nuclei), and the numbers of both 4C and 8C nuclei gradually increased until 18 h after the initiation of COL treatment. After 20 h of COL treatment, the numbers of 4C and 8C nuclei transiently decreased, indicating that a large number of cells were at the prometaphase to metaphase stages. These results indicate that FCM analyses offer a simple and rapid means of preliminary examination for arresting cell-cycle at metaphase in partially synchronized suspension cultures of *H. hybrida*.

Key words: flow cytometry, *Hemerocallis hybrida*, mitotic index, synchronization of cell division

We aim to produce transgenic plants with one or a few alien chromosomes via microprotoplast fusion (Verhoeven *et al.*, 1991; Ramulu *et al.*, 1995; Binsfeld *et al.*, 2000) for genetic improvement and chromosome studies in the Liliaceous ornamentals including *Hemerocallis hybrida*. For applying this technique, it is prerequisite to establish an efficient system for mass-preparation of metaphase-arrested cells through synchronizing cell division by the sequential treatment of cells with a DNA synthesis inhibitor and a spindle toxin. To evaluate synchronizing cell division to metaphase, periodic observations of cell squashes under a microscope is generally needed during the treatment with a spindle toxin. However, this process is rather complicated and time-consuming, and it is desirable to apply an alternative method. In the present study, therefore, we examined the applicability of a FCM analysis for evaluating synchronization of cell division in suspension cultures of *H. hybrida*.

Suspension cultures of *H. hybrida* cv. Stella d'Oro (2n=2x=22), which consisted of fine cell clumps and showed fast growth, were used in the present study. The cultures were initiated from calli, which had been induced from root segments of *in vitro*-grown plantlets on MS (Murashige and Skoog, 1962) medium containing 10 mg l^{-1} picloram, 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum, and maintained using the same medium but lacking gellan gum at 25 ℃ in the dark on a rotary shaker (100 cycle min^{-1}) (Saito and Nakano, 2000). They were subcultured every three days and about a 2.5-fold increase in packed cell volume was obtained in three days. For partial synchronization of cell division, suspension cells were sequentially treated with

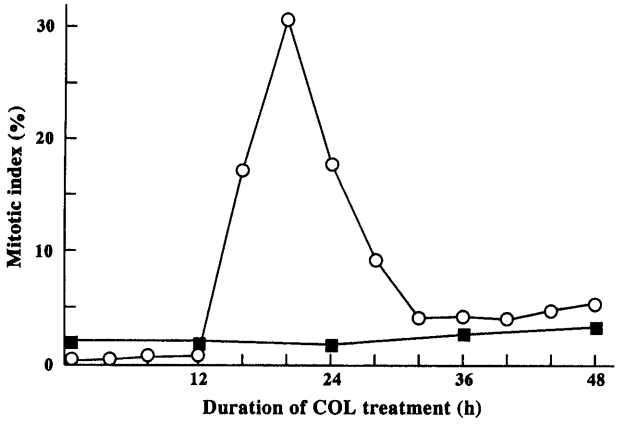

Fig. 1 Time courses of MI after the initiation of COL treatment in suspension cultures of *Hemerocallis hybrida* cv. Stella d'Oro. ○, 250 μM COL; ■, without synchronization treatment (control).

HU (Sigma Chemical Co., St. Louis, Mo., USA) and COL (Kanto Chemical Ltd., Tokyo, Japan) as follows: HU at a final concentration of 2 mM was added to the suspension cultures 12 h after subculture; after 24 h of HU treatment the cells were washed three times with HU-free fresh medium; and then were re-cultured in a medium containing 250 μM COL. During COL treatment, the suspension cells were periodically analyzed for the relative fluorescence intensity of their nuclei by using a FCM (PA; PERTECH, GmbH, Münster, Germany). Cells (ca. 0.1 g FW) were chopped in 0.5 m*l* Solution A (Partec GmbH kit) for isolating nuclei and then stained for 10 min with 2 m*l* of Solution B (Partec GmbH kit) containing 4,6-diamidino-2-phenylindole (DAPI) prior to flow cytometric analyses. At the same time, the cells were squashed under a cover glass, stained with 1% acetocarmine, and observed under a microscope to determinine MI.

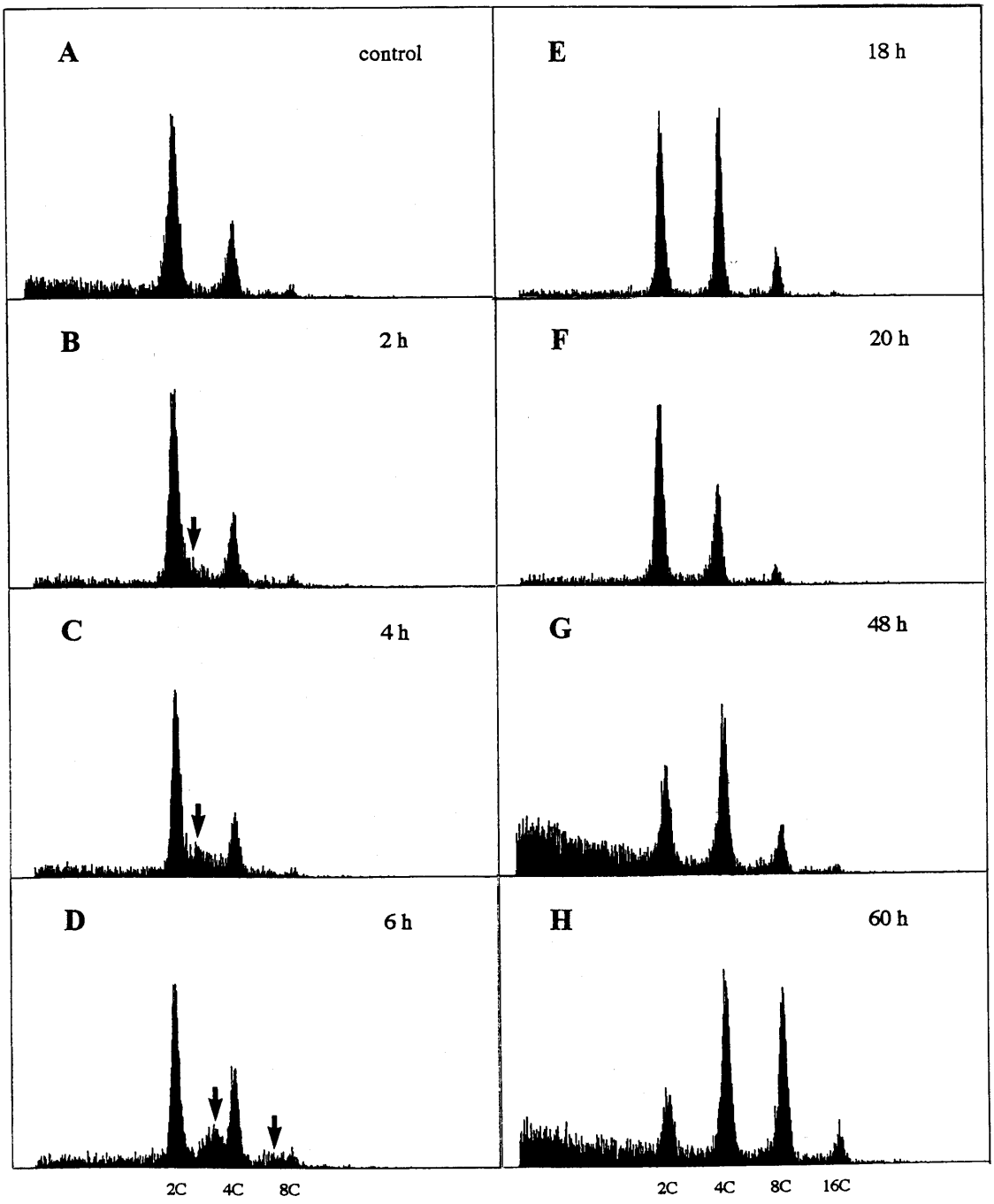

Fig. 2A-H Histograms from FCM analyses of nuclear DNA content of suspension-cultured cells of *Hemerocallis hybrida* cv. Stella d'Oro after the initiation of COL treatment. DNA content is expressed as the fluorescence intensity and C-value on the horizontal axis, and the number of nuclei on the vertical axis. (A) Without synchronization treatment (control). (B)-(H) After 2, 4, 6, 18, 20, 48 and 60 h, respectively. Arrows represent nuclei at S-phase of cell cycle.

Fig. 1 shows the time course of MI after the addition of COL. In the control, asynchronous cultures without both HU and COL treatments, MI was constantly 2 - 3% during the 3-day culture period. On the other hand, MI started to increase suddenly 16 h after the initiation of COL treatment, reached a peak (30.5%) after 20 h, and decreased thereafter.

Histograms of relative fluorescence intensity of nuclei obtained from FCM analyses are shown in **Fig. 2**. In the asynchronous cultures, the histogram had three peaks, corresponding to 2C, 4C and 8C DNA contents, respectively, indicating that the cultures consisted of cells with different ploidy levels (**Fig. 2A**). Nuclei at S-phase of the cell-cycle (DNA replication phase), which are expected to lie between the peaks of the histogram, were scarcely observed in these cultures. No apparent changes in the histograms were observed in the asynchronous cultures throughout the 3-day culture period. In the partially synchronized cultures, the number of S-phase nuclei started to increase 2 h after the initiation of COL treatment, and two novel peaks of S-phase nuclei appeared after 6 h (arrows; **Fig. 2B-D**), indicating that a large number of cells started to replicate DNAs upon release from HU treatment. Both peaks of S-phase nuclei disappeared thereafter, and the numbers of both 4C and 8C nuclei increased until 18 h after the initiation of COL treatment (**Fig. 2E**). These results indicate that the cells progressed via S-phase to G2-phase of the cell-cycle. After 20 - 22 h of COL treatment, the numbers of 4C and 8C nuclei transiently decreased (**Fig. 2F**), probably due to the progression of a certain number of cells into M-phase in which the nuclear membrane disappears at prometaphase. After that the numbers of 4C and 8C nuclei increased again, and a novel peak of 16C nuclei appeared at 60 h after the initiation of COL treatment (**Fig. 2G, H**). Such increases in the number of 4C, 8C and 16C nuclei may have resulted from chromosome doubling via inhibition of spindle organization by COL treatment (Jensen, 1974). After 48 and 60 h of COL treatment, the population showing DNA content below 2C level increased, which might be nuclear debris resulting from prolonged treatment with COL.

In the present study, progression of the cell-cycle in partially synchronized suspension cultures could be clearly followed by periodic FCM analyses. When the peak of MI was obtained, the number of 4C and 8C nuclei transiently decreased. We conclude that FCM analyses offer a simple and rapid means for preliminary examination of the synchronization of cell division in partially synchronized suspension cultures of *H. hybrida* before microscopic observations of cell squashes.

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