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Molecules and Cells

Flow Cytometric Analysis and Chromosome Sorting of Barley (*Hordeum vulgare* L.)

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Flow cytometric analysis was systematically performed to optimize the concentration and duration of hydroxyurea (DNA synthesis inhibitor) and trifluralin (metaphase blocking reagent) treatments for synchronizing the cell cycle and accumulating metaphase chromosomes in barley root tips. A high metaphase index (76.5% in the root tip meristematic area) was routinely achieved. Seedlings of about 1.0-cm length were treated with 1.25 mM hydroxyurea for 14 h to synchronize the root tip meristem cells at the S/G2 phase. After rinsing with hydroxyurea, the seedlings were incubated in a hydroxyurea-free solution for 2 h and were treated with 1 μM trifluralin for 4 h to accumulate mitotic cells in the metaphase. The consistent high metaphase index depended on the uniform germination of seeds prior to treatment. High-quality and high-quantity isolated metaphase chromosomes were suitable for flow cytometric analysis and sorting. Flow karyotypes of barley chromosomes were established via univariate and bivariate analysis. A variation of flow karyotypes was detected among barley lines. Two single chromosome types were identified and sorted. Bivariate analysis showed no variation among barley individual chromosomes in AT and GC content.

Keywords: Barley; Chromosome Isolation; Cytometry; Flow Karyotype; Trifluralin.

Introduction

Flow cytometry has been widely used in genome research due to its sensitivity and precision. Flow cytometric studies using isolated metaphase chromosomes have been applied to many fields of molecular cytogenetics and molecular biology research. Flow cytometry can analyze and sort individual chromosomes and can provide chromosome-specific mapping and the construction of chromosome-specific libraries. To date, the construction of chromosome-specific DNA libraries for each of the 24 human chromosomal types has been completed (see references in Van Dilla and Deaven, 1990). In spite of significant progress in human chromosome research using flow cytometry, the application to plant systems is still lacking owing to technical difficulties, including the preparation of high-quality and high-quantity chromosome suspensions suitable for flow cytometric analysis and the isolation of single chromosome types. Nevertheless, since the first report of plant chromosome flow karyotyping in 1984 (de Laat and Blass, 1984), a few groups have succeeded in flow karyotyping and sorting of plant chromosomes (Arumuganathan *et al*., 1991; Conia *et al*., 1987; Dolezel *et al*., 1992; Gualberti *et al*., 1996; Lee *et al*., 1996; 1997; 1999; Wang *et al*., 1992).

The successful flow karyotyping and sorting of individual chromosomes requires the preparation of high-quality metaphase chromosome suspensions in large quantities. Accumulation of large numbers of metaphase chromosomes in root tips is difficult and depends greatly on precise laboratory experiment. To date, flow karyotyping and sorting of chromosomes isolated from root tip meristemic cells have been performed in *Vicia faba* (Dolezel *et al*., 1992), pea (Gualberti *et al*., 1996), maize (Lee *et al*., 1996), wheat (Lee *et al*., 1997a; 1997b), and rice (Lee *et al*., 1999).

The synchronization of the cell cycle in barley root tips has been achieved by treatment with cold water, DNA-synthesis inhibitors, and metaphase blocking reagents. Pan *et al*. (1993) reported that a metaphase index of 52.0% was possible in barley root tips using sequential treatments of hydroxyurea (Hu), colchicine, and cold water; however, the procedure needs to be

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systematically optimized. The optimal parameters for a high metaphase index suitable for flow cytometric analysis are different for seedling sizes, the duration of chemical treatment, cell types, and each species (Lee *et al.*, 1996; 1997a; 1997b; 1999).

In this article we describe an optimal procedure for cell cycle synchronization and intact mitotic chromosome isolation from barley root tips. Chromosome suspensions isolated from barley root tips were suitable for flow cytometric analysis in quantity and quality. The variation of flow karyotypes was detected among barley lines. Two single chromosome types were identified and sorted. Isolated chromosomes were analyzed by univariate and bivariate karyotyping.

Materials and Methods

Plant materials Barley normal lines, NE 86954, Betzes (kindly provided by Dr. Baenziger, University of Nebraska-Lincoln, USA), Elgina, Frigga, ST 13559, Trumpf (kindly provided by Dr. Kunzel, Gatersleben, Germany), and chromosome 6 trisomic line (kindly provided by Dr. Hang, USDA-ARS, ID, USA) were used in this study.

Cell-cycle synchronization The synchronization of the cell cycle in barley root tips was performed as described by Lee *et al.* (1996). Barley seeds were germinated on moistened paper towels for 30 h. Seedlings with about 0.8–1.0-cm primary roots were treated with 0, 0.5, 1.0, 1.25, 1.5, and 2.0 mM Hu (DNA synthesis inhibitor, Sigma) in 10 ml Hoagland's solution (Sigma) and were incubated at room temperature in the dark. The terminal 1.5 mm of the root tips from the primary roots of five seedlings were analyzed at 1-h intervals from 0 to 24 h for each Hu treatment using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) to determine the percentage of nuclei in the G1, S, and G2/M phases.

Trifluralin (metaphase blocking reagent, gift from DowElanco, Midland, MI, USA) was used for accumulating large numbers of root tip cells in the metaphase. Barley seedlings treated with 1.25 mM Hu for 14 h were rinsed three times in distilled water, incubated in Hu-free Hoagland's solution for 2 h (root tip cells in late S or early G2/M phase), and then treated with trifluralin of 0.1, 0.5, 1.0, and 1.5 μM in 10 ml Hoagland's solution. The terminal 1.5 mm of the primary root tips was analyzed at 1-h intervals, and the optimal trifluralin concentration and treatment duration were determined on the basis of observed metaphase indices, the qualitative evaluation of chromosome morphology, and flow karyotyping. All treatments were performed in the dark at room temperature. After trifluralin treatment for 4 h, the seedlings were placed in ice–water for 18 h for analyses of flow karyotyping and the metaphase index.

The mitotic index was determined by scoring at least 100 cells per root tip (0.5 mm from root cap) selected randomly. The mean mitotic index was calculated from 20 observations.

Chromosome isolation Chromosome suspensions were prepared as described previously (Lee *et al.*, 1996). Briefly, meristem tips (1–1.5 mm) of ten synchronized barley roots were chopped with a sharp, sterile scalpel blade in 0.5-ml ice-cold chromosome isolation buffer (10 mM $MgSO_4 \cdot 7H_2O$, 50 mM KCl, 5 mM Hepes, 3 mM dithiothreitol, 0.25% Triton-X 100) in a petri dish. The chromosome suspension was filtered through a 30-μm nylon mesh.

Univariate flow cytometric analysis For univariate flow karyotype analysis, propidium iodide (PI) was added to the isolated chromosome suspension to a final concentration of 25 μg/ml and the mixture was then incubated on ice for 30 min. The chromosome suspension was analyzed on a FACScan flow cytometer with deionized water as the sheath fluid and with a low sample flow rate of about 12 μl/min sample through the flow cell. The excitation source was an argon ion laser emitting a 488-nm beam at 15 mW for excitation of PI. Red PI fluorescence was collected with a standard 585/42-nm band-pass filter in the FL2 channel and with a 650-nm long-pass filter in the FL3 channel. Forward light scatter values, on a linear scale of 1,024 channels, and PI-fluorescence intensities (FL3 peak height), on a logarithmic scale of fluorescence of four decades, were measured for all particles in the chromosome suspensions. The PI-fluorescence pulse area (FL2-A) was measured on a linear scale of 1,024 channels for barley chromosomes in the preparations. The data were collected and were analyzed with CellQuest software (Becton Dickinson, San Jose, CA, USA).

A theoretical monoparametric flow karyotype was constructed according to Conia *et al.* (1989). The published relative chromosome size (Bennett and Smith, 1976) was used for this purpose. Channel numbers and the frequency values corresponding to the channel numbers were used to draw the theoretical flow karyotype.

Bivariate flow cytometric analysis For bivariate flow cytometric analysis, the chromosomes were stained with Hoechst 33258 (HO, 2.5 μg/ml, Calbiochem, CA, USA) and Chromomycin A3 (CA, 50 μg/ml, Sigma, MO, USA) The bivariate analysis was performed using a FACS Vantage cell sorter system (Becton Dickinson, San Jose, CA, USA). The analysis of the chromosomes was performed at least 2 h after staining to allow the equilibration of the dyes. The primary laser was turned to 200 mW of 457 nm for excitation of CA. The secondary laser was tuned to 200 mW of 358–361 nm for excitation of HO. Chromomycin fluorescence was collected with a 475-nm long-pass filter in front of an FL1 photomultiplier tube (PMT). Hoechst fluorescence was collected with a 390-nm long-pass filter in front of an FL4 PMT. Fluorescence area signals (FL1-A and FL4-A) were used to generate the bivariate flow karyotype of barley using CellQuest software. At least 10000 chromosomes were analyzed to generate the bivariate flow karyotype.

Chromosome sorting Chromosome sorting was conducted on a FACS Vantage cell sorter system. Autoclaved chromosome isolation buffer without dithiothreitol was used as a sheath fluid. Sorting gates were set on each of the prominent peaks of univariate or bivariate flow karyotype, in turn, to identify the objects corresponding to them. Objects from the selected peak area were collected directly onto a piece of black nitrocellulose membrane (Millipore Type AA, pore size 0.8 μm) placed on a microscope slide. Chromosomes sorted from each peak were

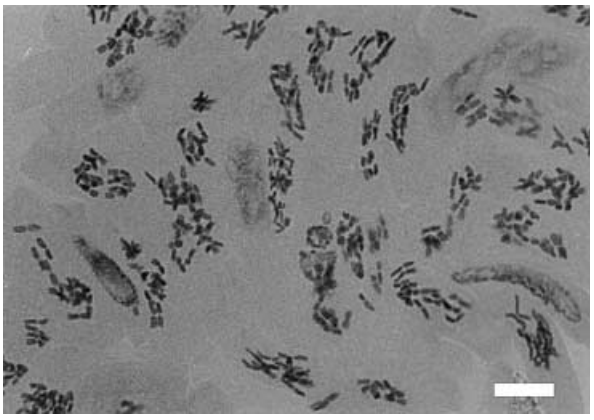

Fig. 1. Accumulated metaphase cells from barley root tips (NE 86954). Seedlings of 1-cm length were treated with 1.25 mM hydroxyurea for 14 h, incubated in hydroxyurea-free Hoagland solution for 2 h, and then treated with 1 μM trifluralin for 4 h. Scale bar = 20 μm.

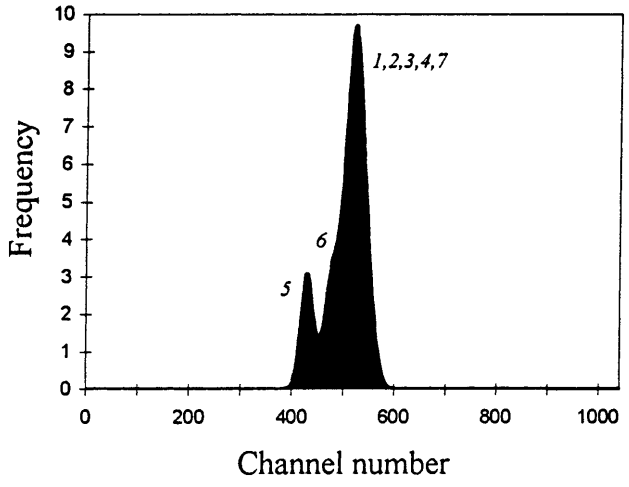

Fig. 2. Theoretical flow karyotypes of barley. Theoretical flow karyotypes were constructed on the basis of the relative chromosome size (Bennett and Smith, 1976) using 4% CV.

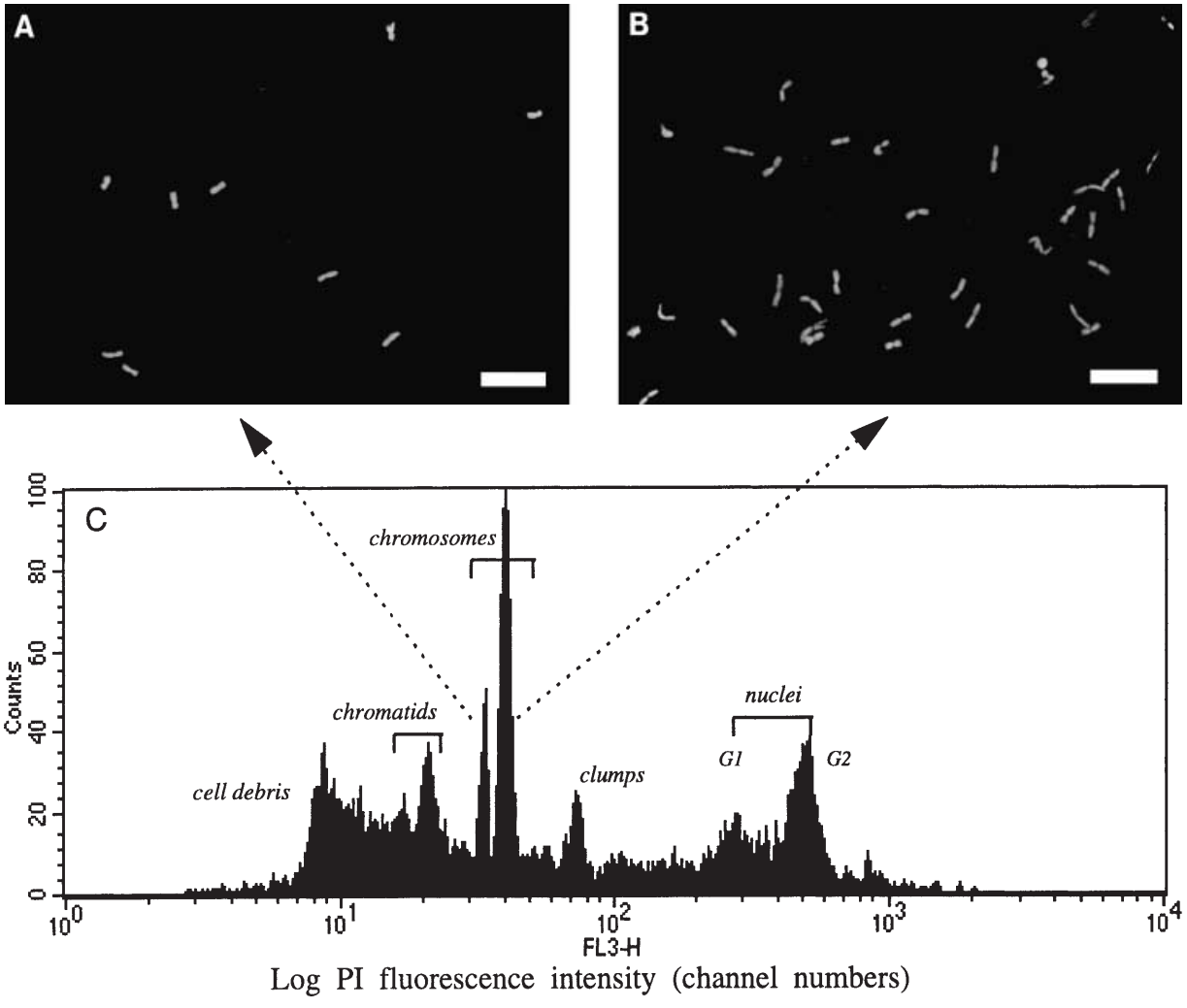

Fig. 3. High-resolution flow karyotyping of barley chromosomes (NE 86954). The photographs show sorted chromosomes from a small peak (**A**) and a large peak (**B**). Relative propidium iodide fluorescence intensity on a logarithmic scale, showing G1 and G2/M nuclei, chromosome clumps, chromosomes, chromatids, and cell debris (**C**). Scale bar = 10 μm.

also collected into microfuge tubes by flow-sorting from well-resolved peaks in the flow karyotype.

To confirm the content of sorted fractions, about 20 µl of dye solution (25 µg/ml PI in chromosome isolation buffer) was added to the sorted fraction on the black membrane, covered with a coverslip, and observed under an Olympus BM60 fluorescence research microscope. Photographs were taken with an Olympus PM30 camera system using Kodak technical pan 2415 black-and-white film.

Results and Discussion

Cell-cycle synchronization and metaphase chromosome accumulation The highly effective synchronization of the cell cycle in barley root tip meristem was achieved by optimizing the concentration and duration of the cell cycle inhibiting reagents, Hu (DNA synthesis inhibitor), and trifluralin (metaphase blocking agent). In previous study, Pan *et al.* (1993) obtaineda metaphase index of up to 52.0% in barley root tips with 1.25 mM HU treatment for 18 h, incubation in HU-free solution for 5 h, 1.25 mM colchicine treatment for 4 h, and ice-cold water treatment for 24 h. Recently, Lee *et al.* (1996; 1997a; 1997b) found that trifluralin is a better metaphase blocking reagent than amiprophos-methyl or colchicine for accumulating metaphase chromosomes in cereal root tip cells. In this study, we systematically optimized HU and trifluralin treatment using flow cytometric and microscopic analyses as described by Lee *et al.* (1996). We routinely obtained a metaphase index of 76.5% in the meristematic area of the barley root tips (Fig. 1) by treatment with 1.25 mM HU for 14 h, incubation for 2 h after removal of Hu, followed by treatment with 1 µM trifluralin solution for 4 h, and 18 h ice–water incubation. The uniformity of seed germination was one of the critical parameters for obtaining consistent high metaphase indices as already pointed out for other cereals by Lee *et al.* (1996; 1997a; 1997b). The selection of seedlings of 1.0-cm length at the time of HU treatment produced repeatedly high mitotic indices. The HU concentration was optimized on the basis of the highest percentage of G2 phase cells which escaped from blocking DNA synthesis before HU removal. The HU treatment lasted until most of the synchronized meristem cells reached the S/G2 phase. The removal of HU at that time allowed the reduction of the duration of the treatment of the metaphase blocking reagent for obtaining high-quality and high-quantity metaphase chromosomes. Trifluralin treatment for more than 4 h did not improve the mitotic index, while the number of chromosome clumps and chromatids increased. Cell synchronization of barley root tip meristem cells was genotype-independent as we analyzed seven different lines; NE 86954, Betzes, Elgina, Frigga, ST 13559, Trumpf, and chromosome 6 trisomic.

Flow karyotyping and chromosome sorting The theoretical flow karyotypes are usually made on the basis of

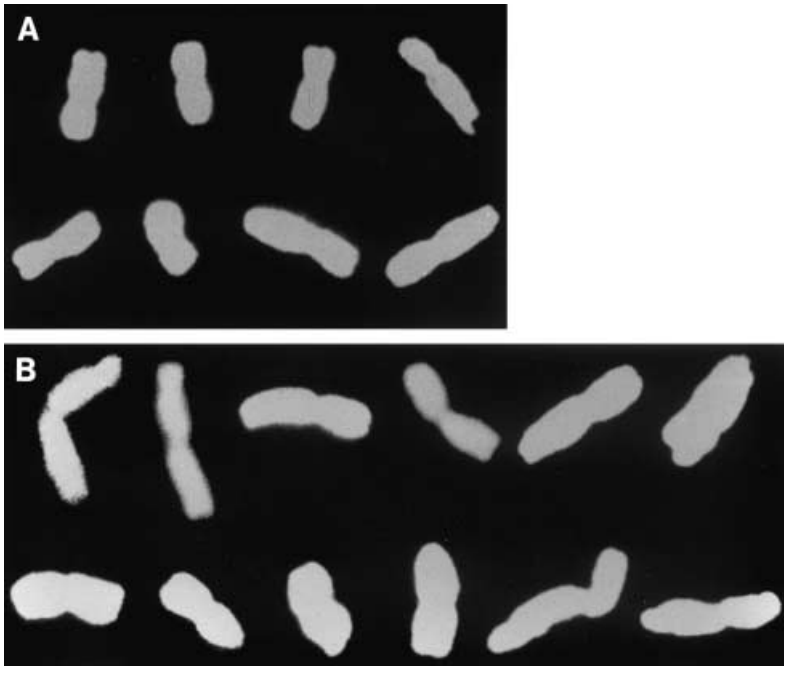

Fig. 4. Flow-sorted barley chromosomes (NE 86954). The chromosomes were sorted on the basis of linear flow karyotypes (Fig. 5A). **A.** Sorted chromosomes from a small chromosome peak. **B.** Sorted chromosomes from a large chromosome peak. A single chromosome type (submetacentric chromosome) was identified from a small peak. A large peak is made of several chromosome types, metacentric, metacentric with secondary construction, submetacentric and submetacentric with secondary construction.

information on relative chromosome sizes (Arumuganathan *et al.*, 1991; Conia *et al.*, 1989; Lee *et al.*, 1996; Lucretti *et al.*, 1993), assuming that the DNA content is uniform along the whole length of a chromosome and among different chromosomes (Schubert *et al.*, 1986). Figure 2 shows a theoretical monoparametric flow karyotype for mitotic metaphase chromosomes of barley based on the published mitotic karyotype (Bennett and Smith, 1976). However, flow cytometric analysis distinguishes chromosomes according to the amount of fluorescence signal received, which, in turn, reflects the DNA content. Lee *et al.* (1997b) found that the DNA content of wheat chromosomes was not necessarily correlated to chromosome size. This result means that the DNA content is not uniform across the whole length of a chromosome nor across a whole genome. Hence, a flow karyotype would better reflect reality if it were constructed using information on the DNA content of metaphase individual chromosomes rather than using relative chromosome size.

Figure 3 shows the high-resolution flow karyotyping of barley metaphase chromosomes based on the relative PI fluorescent intensity. The histogram shows each peak corresponding to nuclei (G1 and G2), chromosome clumps, individual chromosomes, chromatids, and cellular debris (Fig. 3C). Particles from each chromosome peak were sorted and examined under the fluorescence microscope (Figs. 3A and 3B). The morphology of flow-sorted chromosomes was preserved well. One type of chromosome (submetacentric, chromosome 5) was observed in a small chromosome peak (Fig. 4A). Several chromosome types (metacentric, metacentric with secondary construction, submetacentric, submetacentric with secondary construction) were identified in a large chromosome peak (Fig. 4B). However, since the morphologies of the

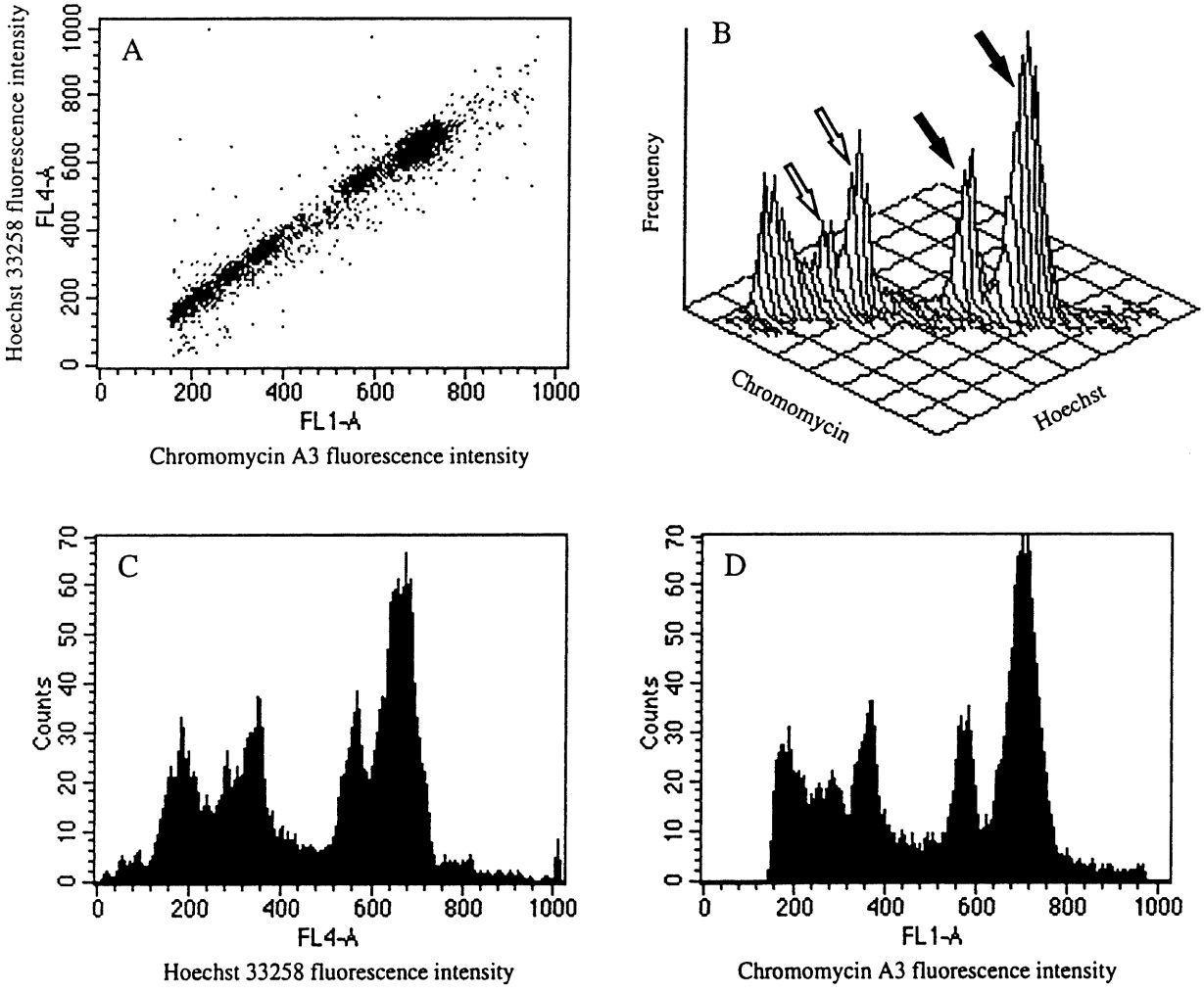

Fig. 5. Bivariate distribution of barley chromosomes (NE 86954) stained with Hoechst 33258 (2.5 μg/ml) and Chromomycin A3 (50 μg/ml). (**A**) Dot-blot; (**B**) Three-dimensional blot; (**C**) Univariate distribution of Hoechst 33258; (**D**) Chromomycin A3. The karyotype is derived from analysis of 10,000 chromosomes. The arrows indicate two groups of chromosomes, and the open arrows indicate two groups of chromatids.

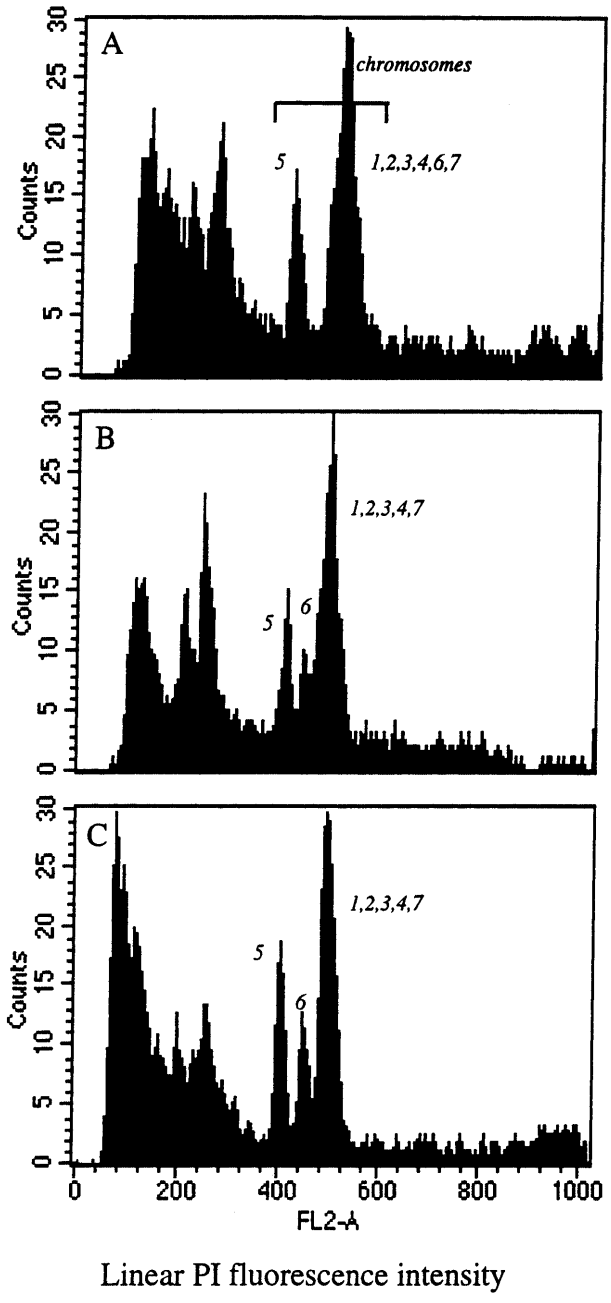

Fig. 6. Univariate distribution of relative propidium iodide fluorescence intensity of chromosomes isolated from normal line, (**A**) NE 86954; (**B**) Elgina; (**C**) chromosome 6 trisomic line. Chromosome 5 and 6 peaks are separated from a composite chromosome peak (**B**, **C**).

barley chromosomes are similar to each other except for the smallest chromosome 5 and chromosomes 6 and 7 with secondary constriction, it is very difficult to distinguish each chromosome using an objective measuring method (Fukui and Kakeda, 1990).

Figure 5 shows the bivariate distribution of barley metaphase chromosomes stained with chromomycin A3 (binds to GC-rich regions) and Hoechst 33258 (binds to AT-rich regions). Two peaks were observed on the bivariate karyotype as for the univariate karyotype. This result indicated that the ratio of GC (chromomycin A3 fluorescence) and AT (Hoechst 33258 fluorescence) content among different barley chromosome types was similar.

As Lucretti *et al.* (1993) pointed out, one obstacle that impedes the application of flow cytogenetics in genome analysis is the inability to resolve single chromosome types on the basis of the flow karyotype. This is also true for barley. From the theoretical flow karyotypes shown in Fig. 2, with the exception of the lowest intensity peaks, which only contain chromosome 5, the other peak represents a mixture of chromosomes with similar DNA content.

Methods must be developed to overcome the obstacle of separating each of the barley chromosome types. The application of aneuploidies, translocations, trisomics, or addition lines in flow-sorting may help solve this problem (Gill *et al.*, 1999). Another potential approach is to collect a single particle corresponding to a composite chromosome peak by flow-sorting into a microtiter plate (one particle per well) and to amplify a single particle to make chromosome-specific DNA probes or chromosome-specific libraries using the PCR method (Telenius *et al.*, 1992; Vandevanter *et al.*, 1994).

Figure 6 shows the variation of flow karyotypes between normal barley lines. The flow karyotype of barley line NE 86954 showed two major chromosome peaks (Fig. 6A), while barley line Elgina produced two major and one minor chromosome peaks (Fig. 6B). This flow karyotype was similar to the barley theoretical flow karyotype (Fig. 2). In the flow karyotype of barley chromosome 6 trisomic line, the chromosome 6 peak was discriminated clearly from the composite chromosome peak (Fig. 6C). In this flow karyotype, two chromosome types, chromosome 5 and 6, could be easily discriminated.

In summary, we have demonstrated an optimized and efficient procedure for the synchronization of cell cycles and the high-resolution flow karyotyping and chromosome sorting from barley root tips. The variability of flow karyotyping in different barley lines will be a useful source for studying the genome organization of barley.

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