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DETERMINATION OF PLOIDY LEVELS OF SHALLOT AND JAPANESE BUNCHING ONION BY FLOW CYTOMETRY

PENENTUAN TINGKAT PLOIDI BAWANG MERAH DAN BAWANG DAUN DENGAN FLOW CYTOMETER

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ABSTRACT

Flow cytometry analysis was successfully used to measure the ploidy of shallot and Japanese bunching onion plants using isolated cell nuclei. In addition, when corrected for ploidy, the nuclear DNA content of shallot cells was found to be twice that of Japanese bunching onion. The chimera phenomenon in tetraploid shallot was also demonstrated.

Key words: flow cytometry, Japanese bunching onion, ploidy, shallot.

INTISARI

Flow cytometer berhasil digunakan dalam menganalisa tingkat ploidy tanaman bawang merah dan bawang daun dengan isolasi inti sel. Selain itu berkaitan dengan tingkat ploidy, kandungan DNA inti sel pada tanaman bawang merah didapatkan dua kali lebih besar dsbanding kandungan DNA inti sel pada tanaman bawang daun. Fenomena chimera juga dapat terlihat pada tanaman bawang merah tetraploid.

Kata kunci: flow cytometry, *tingkat ploidy, bawang merah, bawang daun*

INTRODUCTION

Shallot *(Allium cepa* L. Aggregatum group) and Japanese bunching onion *(Allium fistulosum* L.) are popular edible alliums in the world. Economically the bulbs of shallot and the leaves of Japanese bunching onion are important products. Shallot undergoes active tillering causing the small shallot bulbs to form cluster (Tashiro *et al.*, 1982). At the genetic level shallot has genome AA, $2n = 16$ while Japanese bunching onion has genome FF, $2n = 16$. Because of these facts, extensive crossbreeding studies has been conducted aimed at genetically improving these alliums. It has been well established that the determination of plant genome size is a fundamental parameter to many genetic and molecular studies. A knowledge of haploid nuclear DNA content (C value) is important for basic and applied studies involving genome organization, species relationships, gene expression analysis, and germplasm improvement (Vance *et al.*, 1994). Using haploid shallot and Japanese bunching onion plants as models, we have developed a strategy for increasing the number of standard chromosomes for the production of polyploidy plants. For example we succeeded to obtain polyploid shallot (Endang and Tashiro, 1999) and Japanese bunching onion (unpublished) xpressing haploid ($2n = 8$), doubled haploid ($2n$=16), triploid ($2n = 24$) and tetraploid ($2n = 32$) chromosomes numbers. While the

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tetraploid plants of both alliums were obtained through chromosome doubling of diploid plants using colchicines, the triploid plants were produced by crossing between tetraploid plants and diploid plants.

By chromosome counting and analysis of several phenotypic traits including stomata and epidermal cells sizes we were able to determine the ploidy of haploid, diploid, triploid and tetraploid of shallot and Japanese bunching onion plants (Endang and Tashiro, 1999). However, this way of analysis is technically difficult and time consuming. Recently, flow cytometry has gained much popularity as a method of choices for the rapid and efficient determination of ploidy level in economically important crop (Baird et al., 1994; Dolezel *et al.*, 1994; Dolezel *et al.*, 1989; Ozaki *et al.*, 1998; Lysak *et al.*, 1999). The method has also been used to study of the chimera phenomena chimera in gerbera plants (Tosca *et al.*, 1995). Flow cytometry has potential to replace alternative methods for ploidy screening. This analysis is based on the use of DNA-specific fluorochromes and on the analysis of relative fluorescence intensity emitted by stained nuclei in certain phase of which directly relates to the ploidy level (Dolozel *et al.*, 1994). In this study we evaluated the use of flow cytometry to verify both ploidy levels in shallot and Japanese bunching onion we study flow cytometry.

MATERIALS AND METHODS

Plant materials.
Haploid, doubled haploid, triploid and tetraploid of shallot and Japanese bunching onion plants were used for evaluation flow cytometry analysis.

Determination of ploidy.
The determination of ploidy levels in the allium plants were determined using karyological methods using Feulgen nuclear staining as described by Endang and Tashiro (1999).

Flow cytometry analysis.

Protoplast suspensions were prepared by slicing 0.5 g of newly expanded mature leaf in 1 ml chopping buffer (Arumuganathan and Earle, 1991) containing 10 mM $MgSO_4.7H_20$, 50mM KCl, 50mM Hepes and 0.1% Triton X-100 (pH 8.0). The leaf materials were rinsed thoroughly with distilled water before slicing. A fresh scalpel blade was used for each preparation. The protoplast extracted was broken by passage through Pasteur pipette and the released nuclei were examined and counted under the microscope. The nuclei suspension was filtered (to remove tissue debris and whole cells) through 40μm nylon mesh held in syringe filter unit into microcentrifuge tube. The nuclei were concentrated and washed of any remaining debris by a high-speed centrifugation at 15,000 rpm for 20 seconds. The nuclei pellet was then re-suspended in the chopping buffer. Ethidium bromide (EBr) 1mg/ml was then added to the nuclei suspension so that the final concentration of ethidium bromide in the nuclei suspension was 0.17-0.23 mg/l. Ten μl of ethidium bromide treated nuclei were subjected to flow cytometry. Relative nuclear DNA content was estimated by measuring fluorescent intensity of 10,000 nuclei in each sample.

RESULTS AND DISCUSSIONS

RESULTS

Flow cytometry analysis is based on the use of DNA-specific fluorochromes and on the analysis of relative fluorescence intensity emitted by stained nuclei in certain phase of which related to the ploidy level (Dolozel et al., 1994). In actively growing cell populations the cell cycle can be divided into a series of linked temporal phases. Mitosis(M) is followed by an interval preceding nuclear DNA synthesis (G_1). The period of duplication of the nuclear DNA content (S) is followed by a second interval termed (G_2), the cell then recapitulating to mitosis. Cells with a 2C nuclear DNA content contain the amount of DNA found in G_1-phase diploid cell. Cells with a 4C nuclear DNA content are defined as having an amount of DNA contained in G_2- and M-phase cells. Cells in the S phase have an intemediary DNA content between 2C and 4C (Bourne *et al*, 1989).

The experimental results of the flow cytometry analysis of Japanese bunching onion and shallot are shown in figure 1 to 2. A plot of the relative linear distribution of fluorescence (channel number, on the x-axis) vs. the number of fluorescent events (nuclei counted, on the y-axis) is shown in figure 3. Figure 1 shows the flow cytometer analysis of the Japanese bunching onion in relation to ploidy. Most of the nuclei released from karyologically analyzed haploid, diploid, triploid and tetraploid from Japanese bunching onion plants gave a single peak of relative ethidium bromide fluorescence with relative intensities of 60, 120, 180 and 240 respectively (figure 1; figure 3a). However, the case of shallot plants appears to somewhat different. For example, most of nuclei from karyologically haploid diploid and triploid shallot plants displayed peaks of fluorescence with relative intensities of 180, 280 and 400 of indicative of C, 2C and 3C amounts of DNA content (figure 2; figure 3b). Unlike the The Japanese bunching onion, tetraploid shallot plants were intermediary in chromosomal DNA content with the appearance of two peaks of fluorescence with intensities of 280 and 600, indicative of a 2C and 4C of DNA content. The data clearly demonstrate, for the first time, the chimera phenomena in shallot.

The relationship between ploidy and average fluorescence is shown in figure 3. As can be seen, when corrected for chromosomal ploidy, the DNA content of shallot nuclei was approximately twice that found in the Japanese bunching onion (compare figure 3a with figure 3b). Furthermore, linear correlation between fluorescent intensity and ploidy level in shallot ($r=0.975$, fig.3a) and Japanese bunching onion ($r=0.992$, figure 3b) were recognized.

DISCUSSIONS

Flow cytometry methods designed to estimate nuclear DNA content is a rapid and efficient method for determination of ploidy level in plants. Karyology using Feulgen nuclear staining is a common method to establish chromosome number in root-tip and a relastively economical method. In the cases where high sample numbers need to be examined for the nuclear DNA content, flow cytometry is clearly method of choice over karyology analysis because of automation and speed of analysis. Furthermore, karyology analysis often limited by the low frequency of dividing cells (mitotic index in root is about 1% or less) and the rare occurrence of well spread metaphase plates suitable for chromosome counting (Dolezel et al, 1997).

Flow cytometry analysis is a good tool for the analysis of ploidy level alliums such as shallot and Japanese bunching onion. Furthermore, using this technique we were able to to show chimera phenomena in tetraploid shallot. It has been suggested thas the chimera phenomenoa in Gerbera plants are probably the results of asynchrony of cell divisions (Tosca *et al*, 1995). In that stage nuclei have a relative average DNA content of 2C and 4C. Finallly we found thst nuclei isolated from shallot plants contained significantly higher amounts of DNA than nuclei obtained from the Japanese bunching onion of similar ploidy. This difference may reflect differences in the grouping of species. The linear correlation between fluorescent intensity and ploidy level ($r<0.9$) strongly support the use of flow cytometry as a method of choice for determination of ploidy level in shallot, Japanese bunching onion, as well as other plants of the allium family.

CONCLUSIONS

Flow cytometry analysis was successfully used to measure the ploidy levels of shallot and Japanese bunching onion plants using isolated cell nuclei. The nuclear DNA content of shallot cells was found to be twice that of Japanese bunching onion. The chimera phenomenon in tetraploid shallot was also demonstrated.

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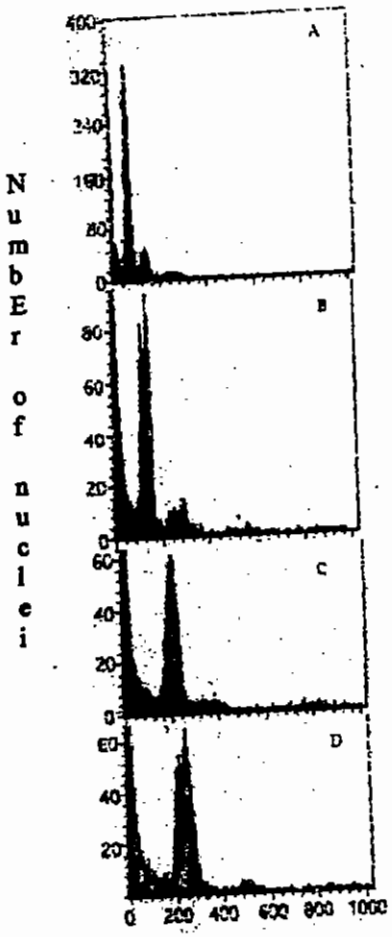

Figure 2. Numbers of nuclei as a function of fluorescence intensity in *A. fistulosum* L. Aggregatum group. A: Haploid, B: Diploid,C: Triploid, D: Tetraploid

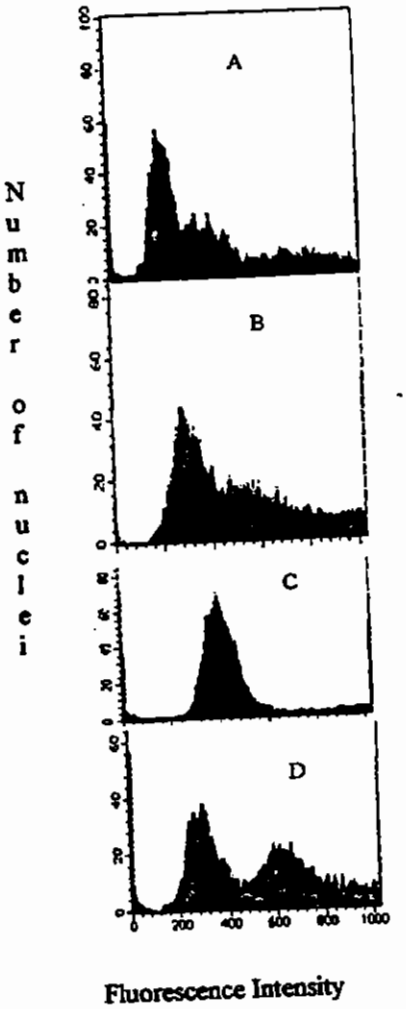

Figure 2. Numbers of nuclei as a function of fluorescence intensity in *A. cepa* L. Aggregatum group. A: Haploid, B: Diploid,C: Triploid, D: Tetraploid

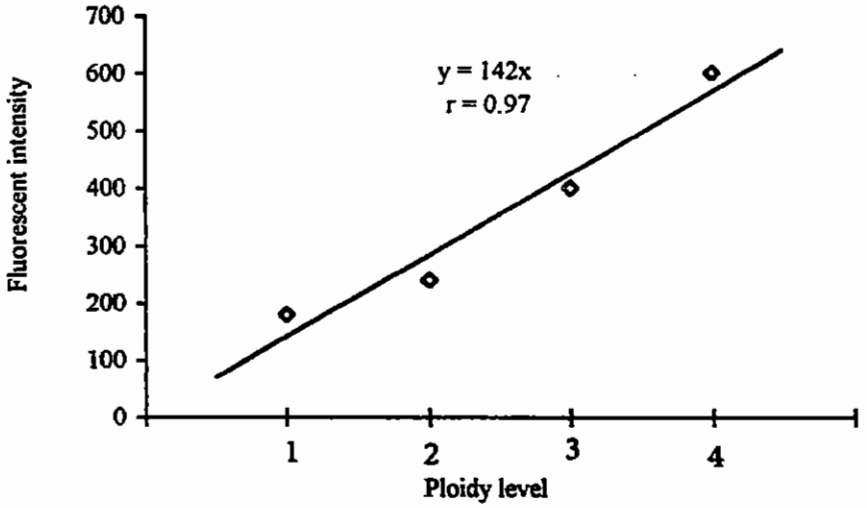

Fig.3a. Relationship between ploidy level and average fluorescent intensity in shallot

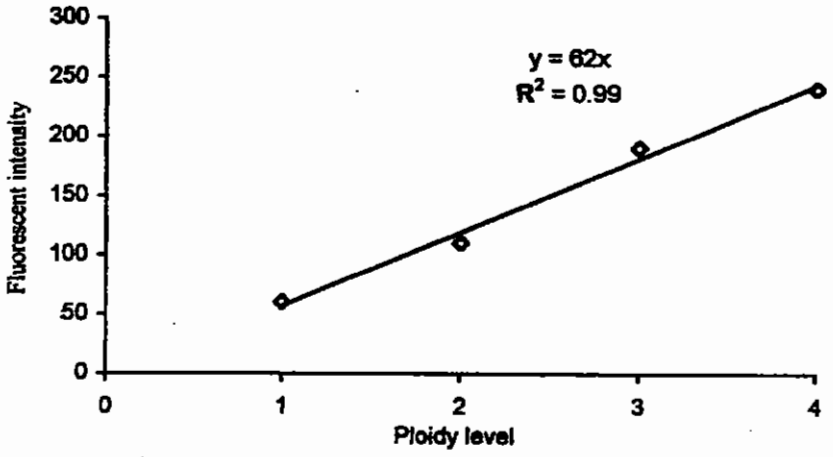

Fig. 3. Relationship between level and average fluorescent Intensity in Japanese bunching onion