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Application of Flow Cytometry for Rapid Determination of Ploidy Levels in Asparagus (*Asparagus officinalis* L.)

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Flow cytometry of PI–stained nuclei was subjected to estimate the ploidy levels in asparagus. No significant difference was observed in PI fluorescent intensity at the prominent peak in diploid cultivars/strain, and there was a very strong positive linear correlation (r=0.9977) between PI fluorescent intensities and ploidy levels in haploid, diploid, triploid and tetraploid cultivars/strains. Flow cytometry has been proven to be a rapid and efficient ploidy analysis during large scale experiments, such as selection of haploid plants from numbers of regenerated plants in anther culture.

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a dioecious perennial species, and originated in Europe and eastern Asia. The dioecious nature of asparagus provides many genetic and physiological studies on sex determination and differentiation. Sex expression has been reported to be governed by only a single locus (Rick and Hanna, 1943), which is located on the L5 chromosome (Löptien, 1979).

Male (XY) plants give higher yields and more vigorous growth than females (XX), and males do not create the 'asparagus weed problem' by producing seeds which germinate and grow in the production fields (Yeager and Scott, 1938). Thus, an all–male population obtainable by crossing with supermales (YY) is one of the major objectives in asparagus breeding. Haploid production by anther culture has been performed to prepare supermale plants (Falavigna *et al.*, 1983; Feng and Wolyn, 1991, 1993). It is, however, difficult to produce haploid plants by anther culture because most of regenerated plants are diploids from somatic cells (e. g., anther wall), not from microspores. The selection of haploid plants is done by counting the somatic chromosomes and by measuring the length of stomata. The procedures are still difficult and/or time consuming to do in asparagus, so that it is necessary to establish the efficient analytical methods for rapid and suitable determination of ploidy levels.

Recently, flow cytometry has become a useful tool for rapid and efficient estimation of genome size and ploidy levels in some crops (Baird *et al.*, 1994; Martínez *et al.*, 1994; O'Brien *et al.*, 1996; Ollitrault–Sammarcelli *et al.*, 1994; Ozias–Akins and Jarret, 1994). The objective of this study is to establish the rapid and efficient method of ploidy determination with flow cytometer in asparagus.

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MATERIALS AND METHODS

Plant materials

Seven diploid cultivars, 'Cito', 'Franklim', 'Geynlim', 'Hokkai 100', 'Larac', 'Mary Washington 500W', and 'UC157F$_1$', one triploid cultivar, 'Hiroshima Green', and one tetraploid cultivar, 'Seto Green', were used in this investigation. One gynogenetic haploid strain, 97SA-003, obtained from the cross between diploid and tetraploid plants (Nakashima *et al.*, 1992), and one supermale diploid strain MM2 obtained from the crosses with hermaphrodite plant, from Hokkaido University, were also used in this study. Number of investigated plants with different genotypes in each cultivar/strain is presented in Table 1.

Flow cytometric analysis

Young cladphylls were cut into pieces in chopping buffer containing 1.0% Triton X-100, 140 mM 2-mercaptoethanol, 50mM Na_2SO_3, 50mM Tris-HCl (pH 7.5) and 25 μg/ml propidium iodide (PI), with a razor for releasing nuclei from cells. The suspension solution was filtered through a 25 μm nylon mesh to remove debris, and the filtrate was centrifuged at 12,000 rpm for one minute. Nuclei in the residue were re-suspended in the chopping buffer, and the suspension solution was subjected to flow cytometric analysis with EPICS XL (Coulter, Tokyo, Japan). Relative nuclear DNA content was estimated by measuring fluorescent intensity of 5,000 nuclei in each sample. The data obtained were analyzed by the XL SYSTEM II (Coulter, Tokyo, Japan).

RESULTS AND DISCUSSIONS

Figure 1 shows a typical result of flow cytometric analysis in diploid asparagus cultivar 'Mary Washington 500 W'. This histogram showed a prominent peak of nuclei at about 300 in PI fluorescent intensity in G_0 and G_1 stages of interphase during the cell cycle. These interphase nuclei in a diploid plant are at the 2C (a double genome) level of DNA content. A minor peak at about twice the intensity value of the prominent peak (about 600) was also distinguished. The minor peak is interpreted as being composed of nuclei primarily in the G_2 stage of interphase and in the early and mid stages of mitosis during the cell cycle. Nuclei in this small peak have an average DNA content of 4C (a quadruple genome) in a diploid. Extensive fluorescent emissions at higher intensities, indicative of populations of nuclei at increased ploidy levels (e. g., 8n, 16n) or nuclear adhesion (e. g., artificial aggregation of individual nuclei forming triplets and quadruplets), were not observed.

Average and range of PI fluorescent intensities at prominent peaks in diploid asparagus cultivars and strain are described in Table 1. The values of individual measurements in diploid plants ranged from 266.0 to 346.0. The variation of average PI fluorescent intensities in each diploid cultivar/strain including the supermale MM2 was not significantly different statistically ($P<0.05$). Average value of all diploid plants was 293.7. Intraspecific variation in DNA content has been reported in *Zea mays*, and a positive correlation between genome size and altitude of the habitat places of the plant was recognized (Rayburn *et al.*, 1989). It was also described that the correlation might be

Table 1. PI fluorescent intensity of the prominent peak in haploid, diploid, triploid and tetraploid cultivars/strains in asparagus.

Ploidy level	Cultivar or strain	No. of plants investigated	PI fluorescent intensity	
			Average	(Range)
x	97SA–003	1	185.0 a	(180.0–190.0)
2x	Cito	3	284.7 b	(278.0–293.0)
	Franklim*	3	301.2 b	(276.0–346.0)
	Geynlim*	3	301.0 b	(286.0–316.0)
	Hokkai 100	3	300.3 b	(296.0–303.0)
	Larac	3	287.3 b	(266.0–319.0)
	Mary Washington 500 W	3	306.0 b	(302.0–314.0)
	UC157F	3	288.7 b	(277.0–295.0)
	MM2**	1	280.0 b	(280.0)
	(Mean in diploids)		293.7	
3x	Hiroshima Green	10	409.3 c	(386.0–428.0)
4x	Seto Green	8	528.1 d	(502.0–562.0)

Mean separation within a column by Duncan's multiple range test (5% level).
* All-male cultivars., ** Supermale strain.

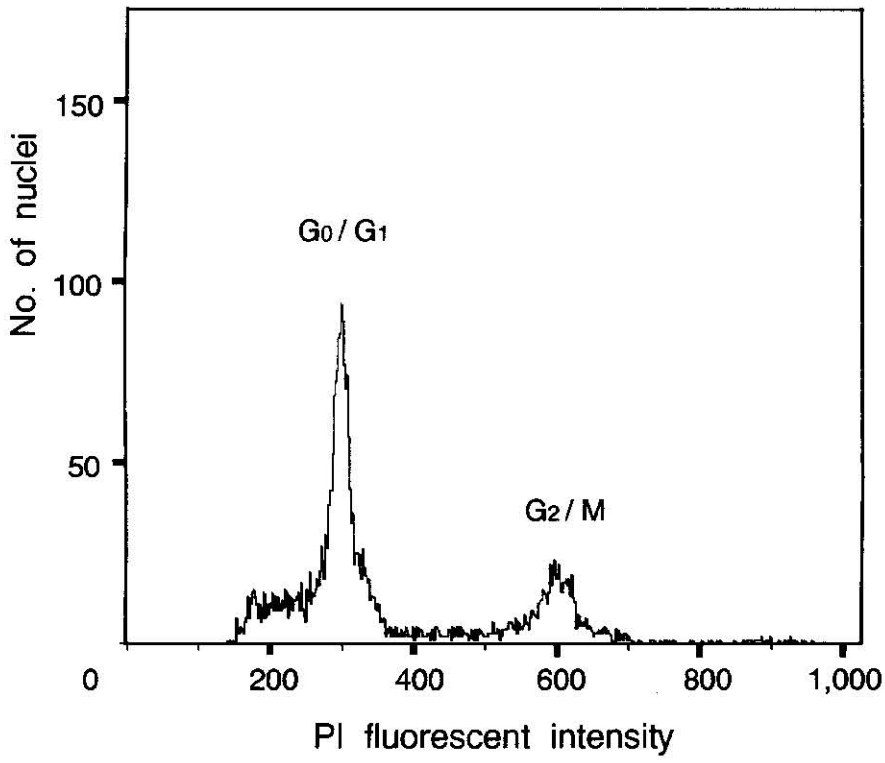

Fig. 1. Flow cytometric histogram pattern in 'Mary Washington 500 W'.

due to increasing knob (C-banded) heterochromatin or additional intra- or supernumerary chromosomal DNA sequences in proportion to increasing altitude (Rayburn and Auger, 1990). In contrast, insignificant variation recognized in diploid asparagus cultivars/strain, proved that there might be little intraspecific variation of genome size in cultivated diploid asparagus.

Silene latifolia (=*Melandrium album*) is one of the model plants as dioecious species (Maegher and Costich, 1994; Veuskens *et al.*, 1992), and sex determinant of the plant was reported to be localized on the strongly heteromorphic chromosome pair (small X and large Y). Flow cytometry is useful for rapid identification of sexes in *Silene latifolia* because of the significant difference in nuclear DNA content between females (2n=24, XX) and males (2n=24, XY) (Dolezel and Göhde, 1995). There are some confused discussions of sex chromosome analysis in asparagus. Although Zilm (1966) and Löptien (1979) reported that asparagus is a homomorphic sex chromosome plant, An *et al.* (1992) and Kitazawa *et al.* (1998) reported it heteromorphic. In the present study, there were no significant differences in flow cytometric data between male (XY) and supermale (YY) plants. The present result supports that the sex chromosome is homomorphic, otherwise the difference in DNA content between X and Y chromosomes might be quite a little if the sex chromosome is heteromorphic in asparagus.

Average and range of PI fluorescent intensities in different ploidy cultivars or strains are also presented in Table 1. The more the ploidy level was from haploid to tetraploid,

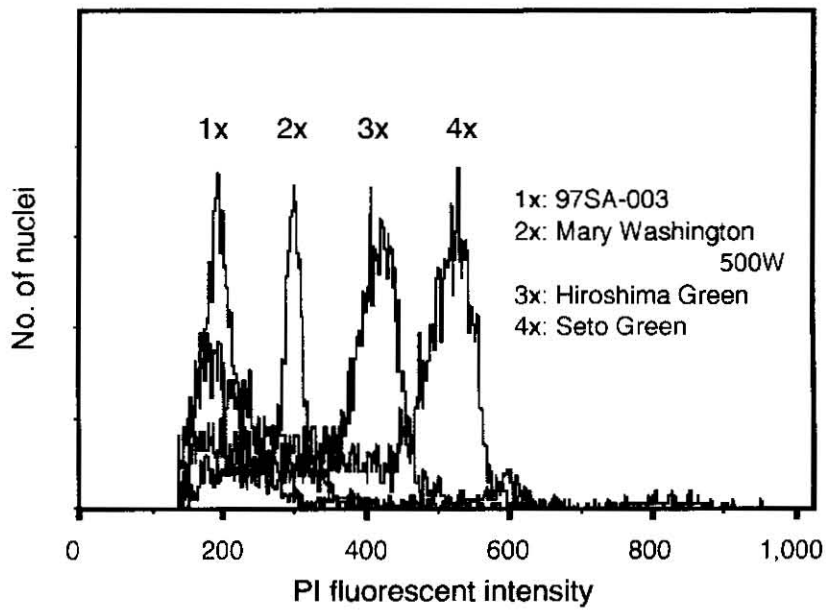

Fig. 2. Flow cytometric histogram patterns in haploid, diploid, triploid and tetraploid plants of asparagus.

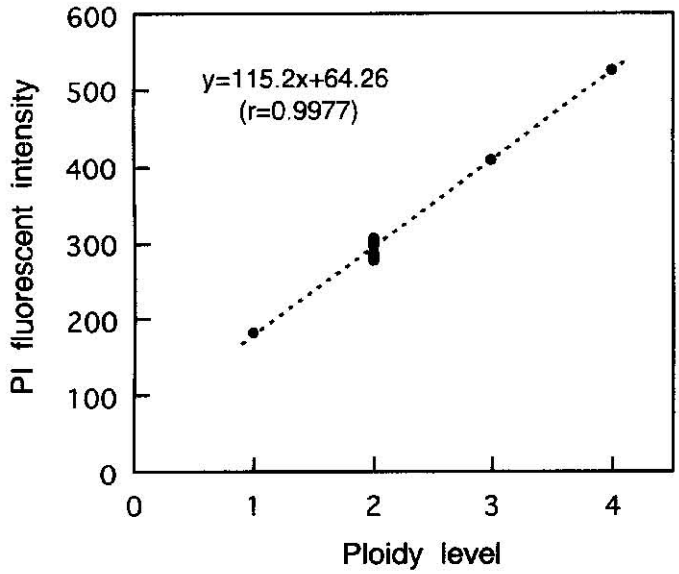

Fig. 3. Relationship between ploidy level and average PI fluorescent intensity in asparagus cultivars/strains.

the larger was the PI fluorescent intensity. Significant differences ($P<0.05$) of PI fluorescent intensities were recognized between different ploidy cultivars/strains. There were no overlapping values in individual measurements between different ploidy plants. Differences in the values of PI fluorescent intensities between haploid and diploid, between diploid and triploid, and between triploid and tetraploid, were approximately the same ranging from 110 to 120 (Table 1 and Fig. 2). Thus, a very strong positive linear correlation ($r=0.9977$) between PI fluorescent intensities and ploidy levels in asparagus was recognized (Fig. 3).

Our study demonstrated that flow cytometry makes rapid and efficient determination of ploidy levels possible without chromosome counting and stomata length measurement in asparagus.

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