Ploidy determination in *Selaginella*

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Abstract

Selaginella (spike mass) is a cosmopolitan genus of spore bearing vascular plants. Like seed, plants, it is eusporangiate, heterosporous and endosporic. Ploidy is a measure of the number of chromosomes in a plant cell. Ploidy can be assessed by chromosomes number or flow cytometry using the DNA index (D1) which is ratio of fluorescence in leukemic blasts compared with normal cells. *Selaginella* is easy to culture and has a much smaller genome than lsoetes. Confirmation of trisomy syndromes is done by cytogenetic techniques. Cytogenetics is the study of the structure and properties of chromosomes, chromosomal behavior during somatic cell division in growth and development (mitosis) and germ cell division in reproduction (meiosis), chromosomal influence on the phenotype and the factors that cause chromosomal changes. Flow cytometer is a fluorescence microscope which analyses moving particles in a suspension is used for determination of polidy. For the fluorescence to be detected by the photomultiplier, the cells have to be labeled with an appropriate fluorescent molecule whose properties will change on binding to nucleic acids. In our studies of immunolabelling there is usually one negative peak (for unlabelled cells) and another one for labelled cells (positive). Such peaks can either be analysed using the (mostly) in-built computer of the flow cytometer, or they can be analysed by drawing ranges or numerical fits to know the mean intensity or the number of cells in a peak. The distribution of the nuclear DNA content within a cell population is obtained by comparing the number of cells in the different peaks or clusters, since the absolute DNA content is irrelevant.

Keywords: *Selaginella*, Ploidy, Chromosomes, DNA index (D1).

Introduction

Ploidy is the number of sets of homologous chromosomes in the genome of a cell or plants. Plant cell with a two set of homologous chromosomes, 2n is described as diploid. Plant cell having multiple sets of paired chromosomes in the genome of an plant is described as polyploidy. Ploidy is a measure of the number of chromosomes in a plant cell. Structures of chromosomes are the thread like structure that contain the genetic material known as DNA[1]. During cell division chromosomes play a key role in the making sure that DNA is copied and distributed capacity. Ploidy can be assessed by chromosomes number or flow cytometry using the DNA index (D1) which is ratio of fluorescence in leukemic blasts compared with normal cells. Generally normal diploid cells have 46 chromosomes and a D1 of 1.0 hyper diploid cell having higher values whereas hypo diploid cells have lower values[2].

During recent years, flow cytometry has established itself as a useful, quick novel method to determine efficiently, reproducibly and at a reduced cost per sample the relative nuclear DNA content and ploidy level of a large number of species (plants and animals), that has also been used to sort cells with different traits from a mixed cell population (e.g. as when separating heterokaryons from parental protoplasts in protoplast fusion experiments for somatic hybrid production) and, more recently, for the analysis of the composition in various chemical components of different tissues (such as apoptotic markers bound to cell compartments). Like seed plants, Selaginella is eusporangiate, heterosporous and endosporic; however, Selaginella produces only spores, not seeds. A similar configuration is found is the related lycopsid isoetes. Heterospory and endospory gametophyte development are found in the leptopsorangiate water habitat pteridophytes[3].

The larger spores are megaspores and the smaller spores are microspores. Megaspores are produced in megasporangia and microspores are produced in microsporangia. Both sporangia are borne in the axils of leaves called microsporophyll and megasporophylls. Megasporangia are present in the basal protion and the microsporangia are present in the upper part of the cone. Each microsporangium contains several microspores. But they are only four megaspores in each sporangium. The mature sores are pyramidal in shape. The sporangial wall consists of three layers. The inner most layer is tapetum. They provide nourishment to the developing spores. A silt is produced in mature sporangia. The spores come out of this slit. The spores germinate to develop gametophytes. Microspore give rise to male gametophytes and the megaspores produces female gametophytes. Both male and female gametophytes remain the walls of the spores. The young embryo develops in the megaspore.

Selaginella is easy to culture and has a much smaller genome than lsoetes. Because of these characteristics, *Selaginella* has been selected as a model for understanding the character transformations that must have occurred in the evolution of seeds. Currently, the genome of *Selaginella moellendorffii* is being sequenced by the Advance study center, Department of Botany Banaras Hindu University, Varanasi, U.P. India. One of the key criteria for selecting *S. moellendorffii* over other *Selaginella* species was its small genome size. At the time *S. moellendorffi* was chosen there were publications detailing the genome size for a total of 6 *Selaginella* species. The Feulgen microdensitometry estimates of Bouchard have generally been disregarded for 2 reasons: (i) the paper that was to include Bouchard's data was withdrawn prior to publication and (ii) the value reported by Bouchard for diploid S. kraussiana is reported to be 2.7 times lower than the corresponding flow cytometric estimate. Thus, only C values obtained by flow cytometry for S. kraussiana and S. moellendorffii were seriously considered. Cultivated S. kraussiana occurs in a polyploidy series (2n = 20, 30, 40)[4].

Confirmation of trisomy syndromes is done by cytogenetic techniques. Cytogenetics is the study of the structure and properties of chromosomes, chromosomal behavior during somatic cell division in growth and development (mitosis) and germ cell division in reproduction (meiosis), chromosomal influence on the phenotype and the factors that cause chromosomal changes. Cytogenetic techniques include karyotyping, fluorescent in situ hybridization techniques and Microarray. For routine analysis, however, the classical karyotyping technique using trypsin and Giemsa became the most accepted worldwide[5]. The banding pattern enabled the detection of various structural aberrations like translocations, inversions, deletions and duplications in addition to the well-known numerical aberrations. Pinkel *et al*., 1986 developed a method to visualize chromosomes using fluorescent-labelled probes called Fluorescent in situ Hybridisation (FISH)[6].

Basically, a flow cytometer is a fluorescence microscope which analyses moving particles in a suspension is used for determination of polidy. These are excited by a source of light (U.V. or laser) and in turn emit an epi-fluorescence which is filtered through a series of dichroic mirrors. Then, the in-built programme of the equipment converts these signals into a graph plotting the intensity of the epi-fluorescence emitted against the count of cells emitting it at a time given. Thus, a flow cytometer consists of fluidics, optics and electronics, as it measures cells in suspension that flow in single-file through an illuminated volume where they scatter light and emit a fluorescence that is collected, filtered and converted to digital values for storage on a computer[3].

Material and Methods

In terms of fluidics, the string of cells in suspension flowing in a single-file is accomplished most frequently by injecting the samples into a sheath fluid as they pass through a small orifice (50-300 µm). Then, when the conditions are right, the sample fluid flows in a central core without mixing with the sheath fluid, and the process is termed "laminar flow". The introduction of a large volume into a small volume in order that it "focuses" along an axis is termed "Hydrodynamic focusing" (Figure-1).

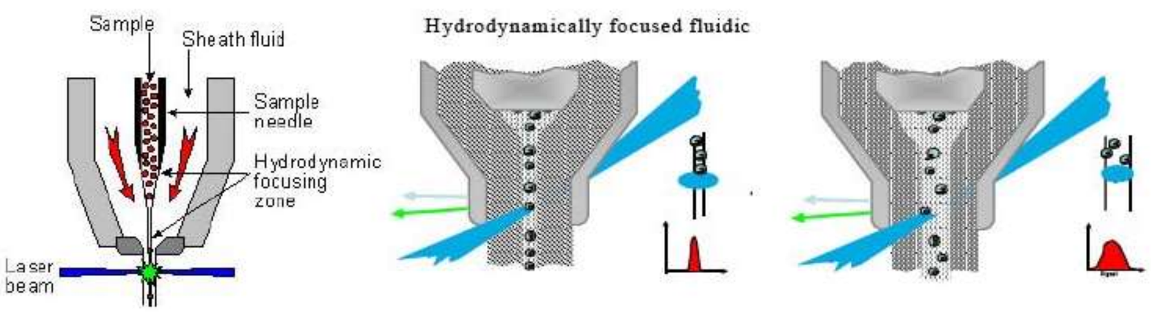

Figure-1: Basics of fluidics in flow cytometry. From left to right, flow chamber, a hydrodynamically focused fluid and an unfocused fluid where the increase of pressure widens the core and increases turbulence (from Murphy, 2006).

In terms of optics, optical signals are generated by the intersection of a light beam with cells or cellular compartments. Two types of light sources can be used, lasers (gas, acid state or diodes) and/or Arc-lamps (mercury or xenon), whereby two different kinds of optical phenomena can be detected by a photomultiplier tube: fluorescence or scattered light[4]. Thus, epilumination by Arc-lamps provides a mixture of wavelengths that must therefore be filtered to select only the desired wavelengths, i.e. it provides incoherent light, but they are inexpensive air-cooled units (Robinson, 2006). Conversely, lasers are monochromatic (i.e. of a single wavelength) and provide coherent light, traditionally from an argon gas source with excitation at 488 nm, but are much more expensive than an UV light source. For the fluorescence to be detected by the photomultiplier, the cells have to be labelled with an appropriate fluorescent molecule whose properties will change on binding to nucleic acids[7].

In fact, the key feature of DNA probes is that they are stoichiometric, whereby the number of molecules of the probe bound is equivalent to the number of molecules of DNA found. Hence, when DAPI for instance binds to the A-T bases of DNA, the intensity of the fluorescence emitted will reflect the number of bounds and therefore also the DNA content in such DAPI-labelled nuclei[8].

Data collection and analysis

In flow cytometry, a parameter is a measured property of the particles, frequently a synonymous to an optical channel. Thus, a one-parameter histogram displays the distribution of cell contents or, in other words, how many cells contain a given quantity of DNA or bind a given number of antibody molecules[9]. In it, such cell content is assigned to one of many classes or channels and is represented on the x-axis, whereas the number of cells being assigned to a given channel is referred to as channel content or simply count and is shown on the y-axis. All cells having about equal quantities of the cell content, e.g. DNA, form a peak. For a typical DNA histogram one peak represents the G1 and another (with twice the channel value) represents the G2/M phase of the cell cycle. In our studies of immunolabelling there is usually one negative peak (for unlabelled cells) and another one for labelled cells (positive). Such peaks can either be analysed using the (mostly) in-built computer of the flow cytometer, or they can be analysed by drawing ranges or numerical fits to know the mean intensity or the number of cells in a peak. On the other hand, in a correlated 2-parameter dot-plot, quantities of the cell properties (such as forward and side scatter intensity) are assigned to channels on the x- and y- axes, where each cell with a given intensity is represented by one dot in the dot-plot[10].

The distribution of the nuclear DNA content within a cell population is obtained by comparing the number of cells in the different peaks or clusters, since the absolute DNA content is irrelevant. Moreover, particular care should be taken to the developmental stage of the analysed cells, as this may alter the quality of the analysis of the cell cycle performed[11]. Also, it is crucial to include an internal standard, as a control, when attempting to analyse the ploidy level of G1-cells by comparing the positions of the first peak in profiles from different genotypes. Frequently used internal standards are chicken erythrocytes, UV Teflon beads, salmon sperm cells or, more simply, a suspension of nuclei of a genotype such as the mother plant (when comparing populations of in vitro regenerants), both parental genotypes (when analysing putative hybrids), or a model system where the relative DNA content per nucleus is well known and reliable among different genotypes within the species of Selaginella. Thus, an internal standard was run together with nuclei suspensions of the genotypes analysed and the position of the G1 peak used to establish the ploidy level of such material[12,13]. In this respect, as explained above, the mean of the first peak will appear at double the intensity of the epi-fluorescence emitted by nuclei of the double population compared to the original one[14].

A second use of flow cytometry is for the analysis of the cell cycle in the nuclei and of the division frequency, expressed through the mitotic index (MI), of the cell population studied[15]. Figure-2, depicts a typical flow cytometry profile for an euploid genotype of Lycopsids and is analysed below.

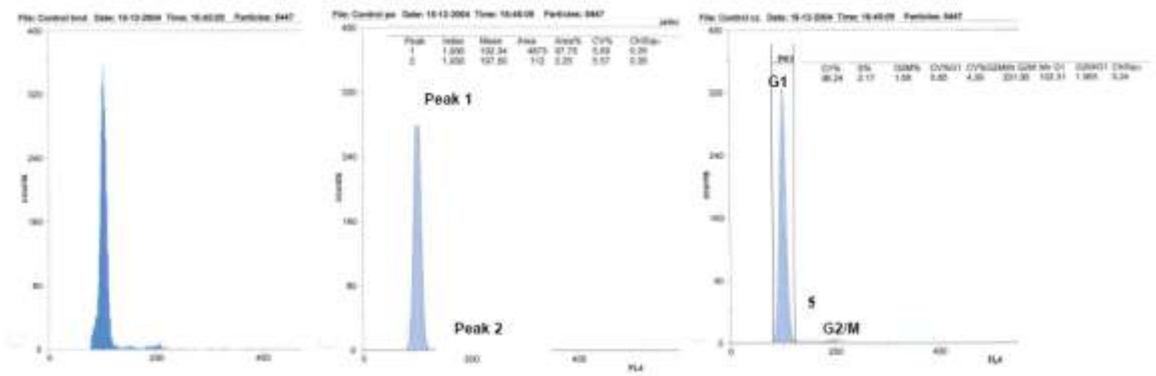

Figure-2: Flow cytometry analysis of a seedling plant of Lycopsids A17. From left to right, the raw profile, peak analysis and cell cycle analysis of a population of 5447 nuclei from leaves.

The mitotic index is calculated, according to the formula,

$$MI = \frac{4x4C}{\Sigma 2C + 4C}$$

where 2C and 4C correspond to the mean value of the first (nuclei in G_1 phase) and second peaks (nuclei in G_2/M phase) in the profiles obtained, respectively. For normal division, MI = 2.000. Very small reductions or increases of the value of the MI indicate problems with cell division and, concomitantly, in the cell cycle (as will be discussed below).

Thereafter, the mean C value of the sample (i.e. the value normally found in the literature) is calculated according to the formula:

$$Mean\,C \quad Value = \sum_{i=1}^{n} \frac{C_1 x\, N_1}{N_{sample}}$$

where N: number of peaks of DNA content in the sample ; C_i: C value of nuclei in peak n_i; Ni: number of nuclei in peak n_i; N_{sample}: number of nuclei in all peaks of the sample[16].

Results and Discussion

A series of experiments was run with materials with a different competence for regeneration in vitro, to check them for ploidy stability, to screen for "x-ploids" and to detect eventual aneuploids and chimaeras. The data in Fig.-2 summarize the range of results observed within this population.

Typical flow cytometry profiles of the major classes of materials produced in vitro barrel medic. Thus, the most frequent, true-to-type regenerates exhibit a two peak profile and coupled with a MI of 2.0. The second most common category of regenerated material concerns the mixoploid calluses. In this case, taking a 2n/4n regenerated individual the profiles are composed of three peaks where the first one corresponds to G1 nuclei of the 2n cell subpopulation, the second peak to the summation of nuclei in G2/M of the same subpopulation plus those in phase G1 of the 4n subpopulation of cells, and the third peak corresponds to the nuclei in phase G2/M of mitosis of the subpopulation of cells of the higher ploidy level. Such material, generally, does not give rise to viable, fertile plants in vivo. Several other classes of tissues can be obtained in vitro and some are also depicted. These include endoreduplicated, aneuploid, senescent and very abnormal tissues[17]. The typical flow cytometric imprint of an endoreduplication phenomenon consists of profiles with a sequence of peaks of progressively decreasing magnitude, which appear as a consequence of the occurrence of a succession of endomitotic DNA replication processes whereby nuclei become bigger and bigger and do not undergo karyodieresis. Such a process leads to the blockage of regeneration competence from such calluses[18].

Conclusion

The summation of these findings makes of flow cytometry an interesting tool for the early prediction of both the regeneration competence from undifferentiated tissues and also of the further fertility of the regenerants obtained. These uses add to the better known utilization of flow cytometry for the characterization of plants, tissues and regenerants in terms of ploidy level, of nuclear DNA content and of division frequency (through the detailed anlaysis of the cell cycles).

References:

1. Rengong M and Chad Finn (2002). Ploidy level and nucler DNA content in Rubus by flow cytometry. J. Amer. Soc. Hort. Sci. 127(5): 767-775.
2. Ochatt SJ, Delaitre C, Lionneton E, Huchette O, Patat-Ochatt EM, Kahane R. (2005) One team, PCMV, and one approach, in vitro biotechnology, for one aim, the breeding of quality plants with a wide array of species. In: Crops Growth, Quality and Biotechnology ISBN 952-91-8601-0 ; ed. R. Dris, WFL Publ. Sci & Technol. Helsinki, Finland.
3. Murphy RF (2006) Basic flow cytometry theory. Flow cytometry talks. Purdue University Cytometry Laboratories.
4. Robinson JP (2006) Introduction to flow cytometry. Flow cytometry talks. Purdue University Cytometry Laboratories.
5. Olson LE, Roper RJ, Sengstaken CL. (2007). Trisomy for the Down syndrome critical region necessary but not sufficient for brain phenotypes of trisomic mice. Hum Mol Genet, 16 : 774-782.
6. Hare WCD, Singh EL (1979). Cytogenetics in animal reproduction. Slough Commonwealth Agricultural Bureaux UK.
7. Pinkel Gray JW, Trask B, Van-den Engh G, Fuscoe J, Van D (1986). Cytogenetic analysis by in situ hybridization with fluorescently labeled nucleicacid probes. Cold Spring Harbor Symb Quant Biol, 51: 151157.
8. Flores-Ramirez F, Guerrero CP, Delgado CG, Morales-Jimenez AB, Arias-Villegas CM, Cervantes A (2015). Cytogenetic profile in 1,921 cases of trisomy 21 syndrome. Arc Med Res., 46:484-489.

9. Belmokhtar F, Belmokhtar R, Kerfouf A (2016). Cytogenetic study of down syndrome in algeria: report and review. J Genet Syndr Gene Ther, 7:280.
10. Dolezel J and Bartos J (2005). Plant DNA flow cytometry and estimation of nuclear genome size. Ann. Bot. (London) 95:99-110.
11. Bennett MD, Leitch IJ, Price HJ and Johnston JS (2003). Comparisons with Caenorhabditis (~100 Mb) and Drosophila (~175 Mb) using flow cytometry show genome size in Arabidopsis to be ~157 Mb and thus ~25% larger than the Arabidopsis genome initiative estimate of ~125 Mb. Ann. Bot. (London), 91: 547-557.
12. Marcon AB, Barros ICL and Guerra M (2005). Variation in chromosome numbers, CMA bands and 45S rDNA sites in species of Selaginella (Pteridophyta). Ann. Bot. (London), 95:271-276.
13. Suda J and Travnicek P (2006). Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry-new prospects for plant research. Cytometry, 69A: 273-280.
14. Wang W, Tanurdzie M, Luo M, Sisneros N, Kim HR, Weng JK, *et al*. (2005). Construction of a bacterial artificial chromosome library from the spikemoss *Selaginella moellendorffii*: a new resource for plant comparative genomics. BMC Plant Biol. 5:10.
15. Webster TR (1979). an artificial crossing technique for *Selaginella*. Am. Fern J. 69:9-13.
16. Smeets DF (2004). Historical prospective of human cytogenetics: from microscope to microarray. Clin Biochem, 37: 439-446.
17. Hulten MA, Jonasson J, Nordgren A, Iwarsson E (2010). Germinal and somatic trisomy 21 mosaicism: how common is it, what are the implications for individual carriers and how does it come about? Curr Genom, 11:409-419.
18. Ochatt SJ, Pontecaille C, Rancillac M (2000). The growth regulators used for bud regeneration and shoot rooting affect the competence for flowering and seed set in regenerated plants of protein pea. In Vitro Cell. Dev. Biol. Plant, 36: 188-193.