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A New less Expensive Method for Genome Size Determination of Plants

B.K. Konwar, D. Chowdhury, J. Buragohain and R. Kandali
Department of Molecular Biology and Biotechnology
Tezpur University, Assam, Pin-784028, India

Abstract: A new method has been developed for easy and inexpensive determination of nuclear DNA content or genome size of plants. This method requires the determination of average cell volume, the number of cells present in a leaf tissue with known weight and intercellular space of the plant species. The nuclear DNA content in a single cell can be calculated easily by dividing the amount of genomic DNA present in a tissue section by the total number of cells in the same tissue. The genome size of five plant species has been determined both by this method and by flow cytometry. The results were found comparable from the accuracy point of view.

Key words: Genome size, method, plant species

INTRODUCTION

Genome size of an organism is the amount of nuclear DNA in its unreplicated gametic nucleus, irrespective of the ploidy level or taxon (Singh, 2003). It is measured by weight or number of base pairs where 1 picogram (pg) equals to 978 megabases (Mb). Hinegardner (1976) described genome size as an important biodiversity character with fundamental significance and wide range of modern biological uses. Till date, genome size of only a fraction of plant species are known (Dolezel and Bartos, 2005). Any breeding program requires information on nuclear DNA content or genome size of the particular plant species. Furthermore, geo-botanical studies may use genome size as an additional parameter for the interpretation of species-specific phenology and the composition of plant communities (Lysak *et al.*, 2000). At present, two most widely used methods for the determination of genome size are Feulgen densitometry and flow cytometry. But, these techniques need sophisticated instruments, which is not possible always in all situations. Although, flow cytometry yields accurate and reproducible determination of 2C DNA content of plant species, the problem starts with expeditions to more distant areas, when the transport and maintenance of the material become an issue. Moreover, the cost of establishing a flow cytometry laboratory may be prohibitive in certain areas (Dolezel and Bartos, 2005). The North Eastern region of the country, regarded as biodiversity hot spot is yet to establish a flow cytometry laboratory. From the above viewpoint, the present investigation has been undertaken to develop an easy, less expensive, accurate and rapid method for the determination of genome size of plants.

MATERIALS AND METHODS

Plant material: Seeds of five medicinal plant species *Streblus asper* Lour, *Spondias pinnata* Kurz., *Zenthoxylum oxyphyllum* Edgew, *Meyna spinosa* Roxb. ex Link and *Rubus alceifolius* Poir were collected and seedlings were raised. Leaf tissues of mature leaves of 60 days old seedlings were taken for the isolation of genomic DNA.

Isolation of plant genomic DNA: Genomic DNA of the plants was isolated from the weighed leaves of the plant species following the procedure described by Khanuja *et al.* (1999) with some modifications. The modifications incorporated in the procedure are overnight isopropanol precipitation, two washings with chloroform: isoamyl alcohol (24:1) and 2 h RNase treatment. DNA quality was determined by measuring absorbance ratio in a Waters UV-Vis spectrophotometer at wavelengths 260:280 nm and then running the DNA aliquots in a 0.8% agarose gel for electrophoresis.

Restriction digestion of the genomic DNA: The isolated genomic DNA was incubated overnight with *Eco* RI and *Hin*d III in the recommended buffer at 37°C and the digested DNA samples were run in a 0.8% agarose gel to check the purity.

Determination of leaf tissue and cell volumes: Fine transverse and longitudinal cross sections of the pre-weighed leaf tissue of 1 cm^2 size were obtained with a sharp razor blade and observed under a microscope (Leica ATC 2000) at 10×40x magnification. The volume of the whole tissue section (1 cm^2) was determined by the

Corresponding Author: B.K. Konwar, Department of Molecular Biology and Biotechnology, Tezpur University, Assam, Pin-784028, India

formula length×breath×thickness. The length, breath and thickness of rectangular cells, length and radius of cylindrical cells and radius of spherical cells were measured with a micro scale having 400x magnifications. Data generated from five randomly selected cells of five random sections as well as cell volumes were recorded with specific formula. The intercellular space of the leaf tissue of the plant was measured in five small leaf sections of known dimensions by measuring with a micro scale at 10×40x magnification.

Genome size determination: The genome size of the plant species was determined with the following formulae

Average volume of a single cell (l×b×t) = $\times\mu^3$ where,
l = length
b = breath
t = thickness
Volume of the tissue (l×b×t) = t μ^3
Volume of the intercellular space = s μ^3
Actual cell mass = (t - s) = v μ^3
Total number of cells in the cell mass = v $\mu^3/\times\mu^3$ = y
Now, weight of the tissue section = w g
w g tissue contains = y cells
So, 1 g tissue will contain cells = y/w
DNA yield per gram of leaf tissue = d μg
= d×10^6 pg
So, one cells contains = (d×10^6)/(y/w) pg
= (d×10^6)/(y/w)×978 Mbp

Flow cytometric analysis of the nuclear DNA: For the isolation of nuclei, the method of Otto (1990) was used with slight modifications. Leaf tissue weighing 20 mg was chopped with a fresh sterile surgical blade in 0.5 mL ice-cold Otto I buffer (0.1 M citric acid monohydrate and 0.5 v% (v/v) Tween 20) in a Petri dish. After chopping another 0.5 mL of ice-cold Otto I buffer was added and mixed well with a pipette. The suspension was filtered through 42 μm nylon mesh and incubated for 15 min. Then added 2 mL of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) followed by propidium iodide (50 μg mL^{-1}) and RNase (50 μg mL^{-1}). The relative DNA content of the isolated nuclei was analysed in a flow cytometer (FAC Scan, working with software the Cell Quest; Becton Dickinson, San Jose, CA) within 15 min. *Pisum sativum* was used as the external reference standard.

RESULTS

The method used by us could yield a considerably high amount of genomic DNA from each of five plant species. The purity of the isolated DNA could be judged from the UV-Vis absorbance ratio at 260: 280 nm and also from the gel electrophoresis of the restriction digested genomic DNA. The yield of genomic DNA per gram of fresh leaf tissue of the selected five medicinal plant species ranged from 34.0 to 48.5 μg having the purity ratio in between 1.77 to 1.89.(Table 1).

The volume of tissue sections of all five plants varied from 1.6×10^{10} to 2.0×10^{10} μm^3. The intercellular space in tissue sections varied between 3.2×10^9 and 6.1×10^9 μm^3. On the other hand, the average volume of single cell ranged from 61,383 to 90,250 μm^3 and the weight of tissue section from 0.02 to 0.03 μg (Table 2).

The value for all five medicinal plants varies between 2.32-3.81 pg or 2.25×10^9 bp-3.72×10^9bp. In the case of flow cytometry, the value ranged from 2.36-3.93 pg or 2.0×10^9-3.93×10^9. Subsequently, the DNA C-value or genome size as determined by the new method and flow cytometry were plotted graphically to assess the variations between the values (Table 3).

Table 1: Yield and quality of the isolated genomic DNA of the plant species

Plant species	Family	DNA yield (μg) g leaf tissue	DNA quality A260 A280
Streblus asper Lour	Moraceae	48.5	1.89
Spondias pinnata Kurz	Anacardiaceae	43.0	1.77
Zanthoxylum oxyphyllum Edgew	Rutaceae	34.0	1.78
Meyna spinosa Roxb. ex Link.	Rubiaceae	46.0	1.86
Rubus alceifolius Poir.	Rosaceae	38.0	1.84

*DNA purest quality = 2.0 (A260: A280)

Table 2: Cell and tissue measurements of the plants studied

Plant species	Volume of the tissue section (tμm^3)	Volume of the intercellular space (sμm^3)	Average volume of single cell (xμm^3)	Weight of tissue section (g)
S. asper	1.6×10^{10}	5.1×10^9	74,490	0.023
S. pinnata	1.75×10^{10}	6.1×10^9	61,383	0.020
Z. oxyphyllum	1.6×10^{10}	3.2×10^9	90,250	0.030
M. spinosa	1.8×10^{10}	3.6×10^9	78,182	0.030
R. alceifolius	2.0×10^{10}	4.0×10^9	76,180	0.030

Table 3: Genome size of the plant species determined by new method and flow cytometry

Plant species	New method 2C-value (bp)	C-value (pg)	C-value (pg)	Flow cytometry 2C-value (pg)	C-value (bp)	C-value (pg)
S. asper	7.62	3.81	3.72×10^9	7.86	3.93	3.84×10^9
S. pinnata	4.64	2.32	2.25×10^9	4.72	2.36	2.30×10^9
Z. oxyphyllum	7.19	3.59	3.51×10^9	7.59	3.79	3.70×10^9
M. spinosa	7.49	3.79	3.70×10^9	7.86	3.93	3.84×10^9
R. alceifolius	5.42	2.71	2.65×10^9	5.68	2.84	2.77×10^9

DISCUSSION

Flow cytometry, Feulgen micro spectrophotometry and DNA image densitometry are the methods employed in the determination of genome size in plants. But, the

methods are found to have some limitations. At present, flow cytometry is becoming a popular method in the determination of genome size. The method needs the establishment of the sophisticated and costly equipment, flow cytometer. The cost is indeed prohibitive for most of the organizations and handling of the equipment needs expert manpower. On the other hand, discrepancies are observed in flow cytometric data of a single sample analysed in different laboratories. Hence, an effort has been made to develop a novel but simple and less expensive method for the determination of genome size of plants without compromising the quality of the work. With the collection of suitable tender leaves from the raised seedlings of the selected medicinal plants for both flow cytometry as well as genomic DNA isolation, we could obtain most of the cell nuclei at G_0/G_1 stage (interphase). This was also supported by Amsellem *et al.* (2001). Genome size of a number crop plants is already known, such as, *Oryza sativa* IR36 (2C = 1.01 pg), *Zea mays* W64A (2C = 5.47 pg) and *Hordeum vulgare* Sultan (2C = 11.12 pg). In this investigation, pea was used as the external standard for the flow cytometric analysis of the isolated cell nuclei as because the nuclear genome of pea is stable (2C-9.09 pg). Moreover, high quality nuclei suspension can be easily prepared from pea leaves appearing to be free of compounds interfering with propidium iodide staining (Baranyi and Greilhuber, 1995). The secondary metabolites commonly present in medicinal plants tend to hamper DNA isolation. However, the protocol followed in the investigation could yield quality DNA (34.0-48.5 μg g^{-1} leaf tissue in the case of medicinal plants studied) as was evident from restriction digestion of the isolated DNA as well as UV-Vis spectrophotometric absorbance ratio at 260: 280 nm being 1.77-1.89 (Table 1). Two critical points were taken into account in this method. Firstly, the method for the isolation of genomic DNA was such that it could isolate almost all the DNA from the nuclei. Secondly, the accurate determination of the intercellular space in the concerned plant species (3.2×10^9-6.1×10^9). The genome size of all five plants determined by both methods has found to be almost similar having a minor variation of 0.04-0.20 pg or 0.5-1.9×10^8 bp (Table 3 and Fig. 1). The developing countries on one hand have a vast resource of plant biodiversity while on the other they face the problem of acquisition and maintenance of flow cytometers (Greilhuber and Temsch, 2001). The method would help in characterizing the vast unexplored plant resources of the developing or underdeveloped countries. The technique of estimating nuclear DNA content of a nucleus using this method is expected to be innovative, unique and first of its kind.

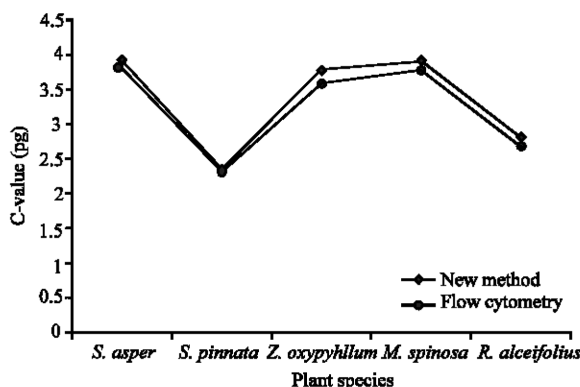

Fig. 1: C-value of five medicinal plant species determined by new method and by flow cytometry

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