Nuclear DNA and Protein Content Evaluation in *Taxus* Plant Cell Cultures Using Multiparameter Flow Cytometry

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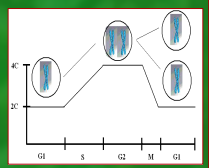
Abstract

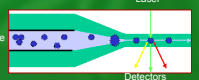
Plant cell cultures of *Taxus* provide the most reliable production methods for the anti-cancer drug paclitaxel. In order to comprehend the inherent culture heterogeneity and production variability in cell cultures, it is essential that the cellular metabolism is studied at the genomic level. Genomic stability in plant cell cultures is crucial as it affects cell growth and division, metabolite accumulation and protein synthesis. A rapid and efficient method to prepare nuclei suspensions from aggregated cell cultures of *Taxus* was employed. Methods were subsequently developed to simultaneously stain them for DNA and protein content using Propidium Iodide and Fluorescein Isothiocyanate respectively. Flow cytometry was used to analyze and quantify the DNA content and genome size of *Taxus* using known reference species as standards. Furthermore, their genomic stability was evaluated by correlating DNA content and genome size with cell size and complexity, protein content, and elicitation effects using multiparameter flow cytometry. These techniques to evaluate and correlate various culture characteristics can be very useful in designing superior bioprocesses for enhanced production.

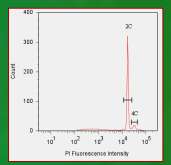
Introduction

Objectives

- To characterize culture heterogeneity through investigation at genomic levels
- To assess the metabolic stability of plant species and understand the progression of cell cycle and DNA content distribution and its control in greater detail through evaluation of DNA content / Genome size and protein content
- To help establish relationships between primary and secondary metabolism to determine the position of the cell within the cell cycle to predict its further development trajectories
- To explain how and under what conditions (DNA content, protein content, cell size, metabolic state, etc.) does the mechanisms governing genomic and metabolic stability get activated







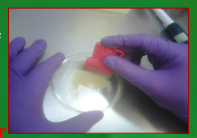
Approach

- Rapid and efficient preparation of nuclei suspensions from aggregated plant cell cultures
- Establishing simultaneous staining protocols for DNA and protein content
- Multiparameter flow cytometric analysis

Process Development

Choice of Nuclei Isolation Method

- Chopping the cells by a razor blade in the presence of a nuclei isolation buffer: fast, cheaper, simpler and radically practical



Choice of Isolation Buffer

Components	Functions	Examples (Concentration used)
Chromatin stabilizer	Minimizes disturbances from complex combination of DNA, RNA, and protein that makes up chromosomes; provides proper ionic strength	$MgCl_2$ (45 mM)
Organic buffer substance	Stabilizes the pH of the solution, usually between 7 and 8	4-morpholinepropanesulfonic acid (MOPS) (20 mM)
Chelating agent	Binds divalent cations, which can serve as nuclease cofactors (non-proteins which can cleave nucleic acids)	sodium citrate (30 mM)
Non-ionic detergent	Releases and cleans nuclei; decreases the aggregation affinities between nuclei and debris without changing the fluorescent properties of the dye molecules	Triton X-100 (0.3 % (v/v))

Choice of Stain/Dye

- **Propidium Iodide (PI)**
  - Intercalates between the bases of the double-stranded DNA (as well as double stranded RNA (dsRNA)) without base-dependent bias
  - Stoichiometric binding (1 dye molecule per 4-5 base pairs of DNA)
  - Best suited for FCM analysis (large Stokes shift and minimal overlap)
  - Simultaneously add RNAse; heated for 15 minutes at 90 °C to render any DNases inactive
- **Fluorescein Isothiocyanate (FITC)**
  - Stains protein (Isothiocyanate is a reactive electrophilic group (-N=C=S) which binds to proteins due to their nucleophilicity)
  - When bound with nuclear proteins, they typically turn fluorescent.

The Procedure

Isolation of Nuclear Suspensions

➢ Cells → vacuum-filter through Miracloth® → transfer 0.5 g to center of a Petri dish → Add ice-cold isolation buffer (1-3 mL) to the Petri dish ➢ Immerse cells in buffer → chop immediately with the mini-glass scraper for five minutes → Mix the resultant sample properly → Transfer to a 15 mL centrifuge tube and add PBS to make up the working volume to 10 mL ➢ Filter the suspension using an 80 µm mesh → divide into 2 parts for staining of nuclear DNA and protein

Staining of Nuclear DNA

➢ Add both PI and Ribonuclease A at 50 µg/mL each and shake the sample properly to disperse the stain → Incubate on ice for 15 minutes with intermittent mixing → Filter again before transferring to 5 mL round-bottom tubes for flow cytometric analysis

Staining of Protein

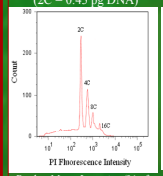
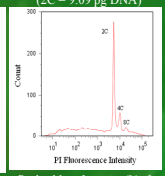
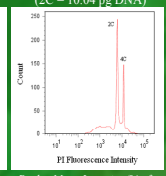
➢ Add FITC at 50 µg/mL and mix thoroughly → Incubate at 4°C for 8 hours and wash to remove the unbound dye → Filter a final time before flow cytometric analysis

Results and Discussion

Plant Reference Standards for DNA content

➢ Use of other plant species with known DNA content (from literature)

➢ Used to evaluate DNA content and Genome Size for *Taxus* cells

Arabidopsis thaliana (2C = 0.43 pg DNA)	*Pisum sativum* (2C = 9.09 pg DNA)	*Nicotiana tabacum* (2C = 10.04 pg DNA)
		
Peaks: Mean Intensity (% of events) 2C: 301 (38.9%) 4C: 586 (29.1%) 8C: 1038 (17.3%) 16C: 2009 (6.7%)	*Peaks: Mean Intensity (% of events)* 2C: 5582 (79.4%) 4C: 9890 (11.4%) 8C: 19660 (3.2%)	*Peaks: Mean Intensity (% of events)* 2C: 6120 (44.3%) 4C: 13008 (39.5%)

DNA content variation in *Taxus* cell lines

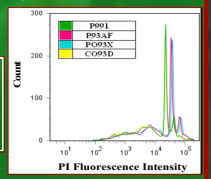
➢ Evaluation of DNA content and Genome Size for four *Taxus* cell lines

➢ Sample 2C value (pg of DNA) = Reference 2C value x (Sample 2C mean peak position / Reference 2C mean peak position)

➢ DNA content ↔ Genome Size:

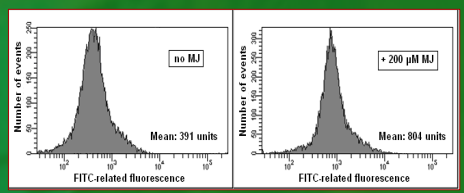
1 pg DNA = 0.978 x 10^9 bp

Cell line	DNA content (pg)	Genome size (bp)
P991	34.62 ± 2.6	3.39E+10
P93AF	53.69 ± 4.1	5.25E+10
PO93X	62.30 ± 4.7	6.09E+10
CO93D	34.81 ± 2.6	3.41E+10



Cell line	2C peak Mean PI intensity	2C peak % of events	4C peak Mean PI intensity	4C peak % of events
Taxus cuspidata P991	22110	43.5	42040	9.4
Taxus cuspidata P93AF	34285	40.1	71450	12.5
Taxus cuspidata PO93X	39787	36.8	78565	11.1
Taxus canadensis CO93D	22236	32.5	43789	8.6

Protein Staining: effect of Methyl Jasmonate (MJ)



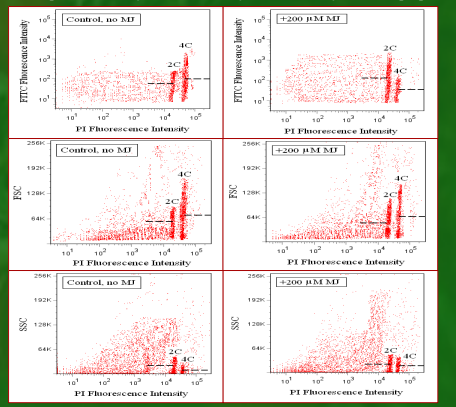
➢ Use of fluorescein diacetate (FITC) to stain for protein

➢ MJ elicited cultures demonstrate > 2-fold increase in protein content over non-elicited cultures

Multiparameter Flow Cytometry Results

➢ Co-staining with PI and FITC for DNA and protein respectively for MJ-elicited and non-elicited cultures

➢ Multiparameter analysis of PI intensity, FITC intensity & scatter properties



Three definable regions (FITC vs. PI staining) -

- 2C: increasing protein content and constant DNA content
- S: constant protein content and increasing DNA content
- 4C: increasing protein content and constant DNA content

Protein content in S phase -

- no MJ: increases from 2C to 4C → histone content increases in proportion to the DNA content
- + MJ: decreases from 2C to 4C → MJ decreases histone availability as a result of up-regulation of secondary metabolism; and MJ induced nuclei result in protein degradation and formation of non-histones

Scatter properties -

- Forward Scatter (FSC): increases as cells synthesize DNA and pass from 2C to 4C → increase in cell size and DNA content
- Side Scatter (SSC): decreases from 2C to 4C → nuclei's inner complexity decreases with decrease in histones availability

Conclusions

- A rapid and simple method was employed to isolate nuclei from aggregated plant cell suspensions
- Simultaneous staining procedures for DNA and protein were developed
- DNA content / Genome size was evaluated using known plant reference species
- Multiparameter flow cytometry was performed to correlate various cellular parameters under different conditions
- Will help establish correlations to control and predict culture behavior in order to optimize bioprocesses for enhanced metabolite production

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Department of Chemical Engineering, University of Massachusetts Amherst