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Flow cytometric analysis of variation in the level of nuclear DNA endoreduplication in the cotyledons amongst *Vigna radiata* cultivars

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Abstract — Most of the differentiated plant cells replicate their nuclear DNA but there is no chromosome condensation, strand separation, nor cytokinesis, resulting in multiple copies of the genome, and the phenomenon is called endoreduplication. In this communication we report the distribution pattern of DNA endoreduplication in the nuclei of the mature cotyledons of six cultivars of *Vigna radiata* (mungbean). It has shown that the maximum ploidy level present in the cotyledonary tissues of *V. radiata* is 64C, where 2C is DNA amount of unreplicated diploid nucleus, except in the cultivar ML 337, where only up to 32C DNA content was noted. Considerable difference in the level of C-value distribution amongst different cultivars of *V. radiata* has been found in the present investigation. This is the first report on flow cytometric analysis showing variation in the level of nuclear endoreduplication amongst the cultivars of a grain legume.

Key words: Cotyledon, endoreduplication, flow cytometry, *Vigna radiata*.

INTRODUCTION

Endoreduplication is a common process accompanying cell differentiation in almost all angiosperms studied (Nagl 1978). As a consequence of DNA-endoreduplication differentiated cells replicate their nuclear DNA but there is no chromosome condensation, strand separation, nor cytokinesis, resulting in multiple copies of the genome. Organs and tissues endowed with endoreduplicated DNA are referred to as polysomatic.

In most cells, the initiation of a round of DNA synthesis and initiation of mitosis are coordinated so that the DNA is replicated exactly once in each division cycle. It was assumed long back by Duncan and Ross (1950) that the cell differentiation in maize endosperm occurs through nuclear enlargement that takes place through chromosome endoreduplication. Later, D'Amato (1989) predicted that the process of endoreduplication might be related to differentiation events such as protein and starch synthesis and storage and accumulation of nucleotides, enzyme activation and hormone synthesis. Recently, Das and Pal (2003) have shown that in *Vigna radiata* (mungbean) endoreduplication is associated with storage protein synthesis. Despite common occurrence of endoreduplicated cells in metabolically active tissues and storage organs, the function and significance is poorly understood (Larkins *et al.* 2000). Incidentally, cotyledons of *V. radiata* is the main storage organ of the dietary proteins and the content is as high as 28% of low flatulence, easily digestible protein.

Dunphy and Newport (1988) analyzed the mechanism of inhibition of mitosis by monitoring the *in vitro* phosphorylation of histone H1. The mechanism that inhibits the amount and activity of M-phase promoting factor (MPF) during mitosis in developing maize endosperm was investigated by Grafi and Larkins (1995) and found that MPF protein phosphorylation activity declined dramatically at the onset of endoreduplication, but the amount of P34 protein (a component of MPF) remained constant. In contrast, the amount and activity of S-phase-related protein kinases increased, suggesting endoreduplication of maize endosperm proceeds as a result of two events, inhibition of MPF and induction of S phase-related kinases.

Flow cytometry (FCM) has been reported to be a suitable tool for rapid and accurate analysis of en-

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doreduplication in plant tissues during development (GALBRAITH *et al*. 1991). GALBRAITH *et al*. (1991) have demonstrated tissue specific endoreduplication in *Arabidopsis thaliana*. KNOWLES *et al*. (1990) studied the endoreduplication of nuclear DNA in the developing maize endosperm. DAS and PAL (2003) reported endoreduplication of nuclear genome during cotyledon development of *V. radiata.* The study enumerated the temporal difference in the endoreduplication pattern of nuclear genome between the two cotyledon types of *V. radiata* Cv. B1. Presence of two distinct types of cotyledon (in relation to their attachment with the embryonal axis) in *V. radiata* has been reported earlier (CHANDRA and PAL 1995). These two cotyledon types, 'Cot' and 'Cot È of the six cultivars investigated, showed differential regeneration response under *in vitro* condition (CHANDRA and PAL 1995). To analyze variations in the level of endoreduplication, if any, within these two cotyledon types the C-value as nuclear DNA contents were analysed separately for 'Cot' and 'Cot È of all these six cultivars of *V. radiata*. In this communication we report the distribution pattern of DNA endoreduplication in the nuclei of the mature cotyledons of six cultivars of *V. radiata*.

MATERIALS AND METHODS

Materials - Vigna radiata (L.) Wilczek cultivars B-1, T-44, PM-2, PIMS-4, TARM-2 and ML-2 were collected from Berhampur Pulse and Oil Research Centre, West-Bengal and Bhabha Atomic Research Centre, Mumbai, India. De-embryonated cotyledons of fully mature seeds were excised. The two cotyledons of any mature seed of *V. radiata* could be identified individually as 'Cot È, which remains firmly attached to the embryonal axis and 'Cot', which is easily separable from the embryonal axis.

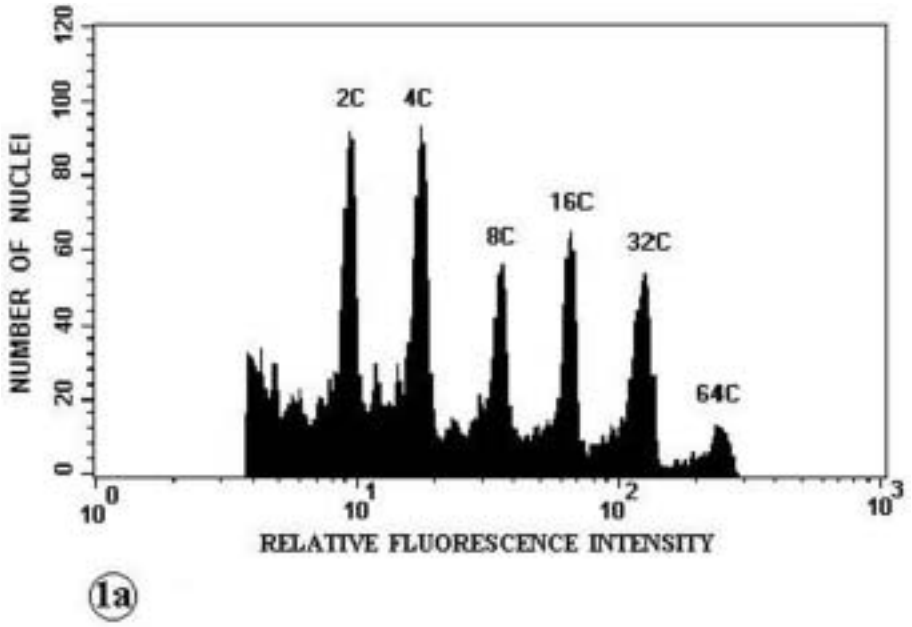
Flow cytometry - Embryos, 'Cot' and 'Cot È from mature seeds of *V. radiata* were excised after imbibition for 20 hr under sterile conditions. Nuclei suspensions were prepared separately from the 'Cot' and 'Cot È of different cultivars of *V. radiata* according to DOLEZ EL and GÖHDE (1995). Approximately 10 mg of all plant tissues were chopped separately using a sharp razor blade with 1.0 ml of ice cold Otto buffer I (OTTO 1990) containing 100mM citric acid and 0.5% (v/v) Tween 20. The nuclei extract was filtered through 50(m nylon mesh and centrifuged at 500*g* for 10 min. The pellet of nuclei was gently resuspended in 500 (l Otto I buffer and kept at 4° C for 30 min. To stain nuclear DNA, 1ml of Otto II buffer (400mM $Na_2HPO_4.12H_2O$) containing 4μg ml^{-1}

Table 1 — The extent of endoreduplication (per cent cells with different C levels) in two types of mature cotyledonary tissues ('Cot' and 'Cot È) of different cultivars of *Vigna radiata*

CULTIVAR		2C	4C	8C	16C	32C	64C
PM-2/Cot	Mean	28.03	26.01	15.63	16.10	12.14	2.08
	SD	3.61	2.17	1.07	2.98	3.33	1.13
PM-2/CotE	*Mean	27.04	23.98	14.77	16.45	15.10	2.64
	SD	2.11	1.32	1.50	0.62	1.77	0.91
T-44/Cot	Mean	23.63	27.47	14.78	14.72	15.99	3.42
	SD	0.72	2.74	0.91	0.50	1.99	1.20
T-44/Cot E	Mean	26.08	27.92	13.41	13.44	13.74	3.41
	SD	3.75	0.31	0.96	0.52	0.90	0.96
TARM-2/Cot	Mean	25.99	34.08	14.57	11.68	11.84	1.79
	SD	2.97	0.41	0.60	1.80	1.29	0.24
TARM-2/Cot E	Mean	21.83	31.64	17.20	13.56	13.08	2.66
	SD	2.53	1.17	1.90	2.02	1.69	0.92
B-1/Cot	Mean	24.72	33.61	17.93	13.06	9.77	0.91
	SD	2.60	1.99	2.34	0.65	1.04	0.13
B-1/Cot E	Mean	26.69	34.97	17.14	11.82	8.55	0.92
	SD	1.10	0.99	1.26	1.30	0.82	0.28
ML-2/Cot	Mean	21.84	32.41	19.94	12.21	13.56	
	SD	5.41	3.61	3.08	1.67	2.96	
ML-2/Cot E	Mean	24.47	30.02	23.81	11.91	9.80	
	SD	2.43	3.35	3.13	1.80	1.29	
PMSE-4/Cot	Mean	23.42	29.50	15.93	12.22	12.54	6.20
	SD	1.26	1.52	2.73	1.26	2.08	1.79
PMSE-4/Cot E	Mean	21.96	25.53	15.28	13.76	16.01	7.10
	SD	2.27	0.64	2.14	2.43	1.11	1.86

* Mean and standard deviation (SD) of three replicate samples.

4'6-diamidino-2-phenylindole (DAPI) as fluorochrome was added to the suspension. DAPI is a nonintercalating stain that binds preferentially and in a complex manner to A-T rich regions of DNA (COLEMAN *et al.* 1981). Stained nuclei were analysed using FACSVantage (Becton Dickinson, USA) flow cytometer equipped with UV laser set to multiline UV and 300mW output power. DAPI fluorescence was measured through a 424/44 band-pass filter in front of fluorescence 1 (FL1) detector. Relative fluorescence intensities, which corresponded to relative DNA content of gated populations, were acquired on histograms of FL1 pulse height. In order to accommodate the broad range of DNA contents, logarithmic signal amplification was used. Data on a total of 10,000 nuclei were acquired for fluorescence measurement.



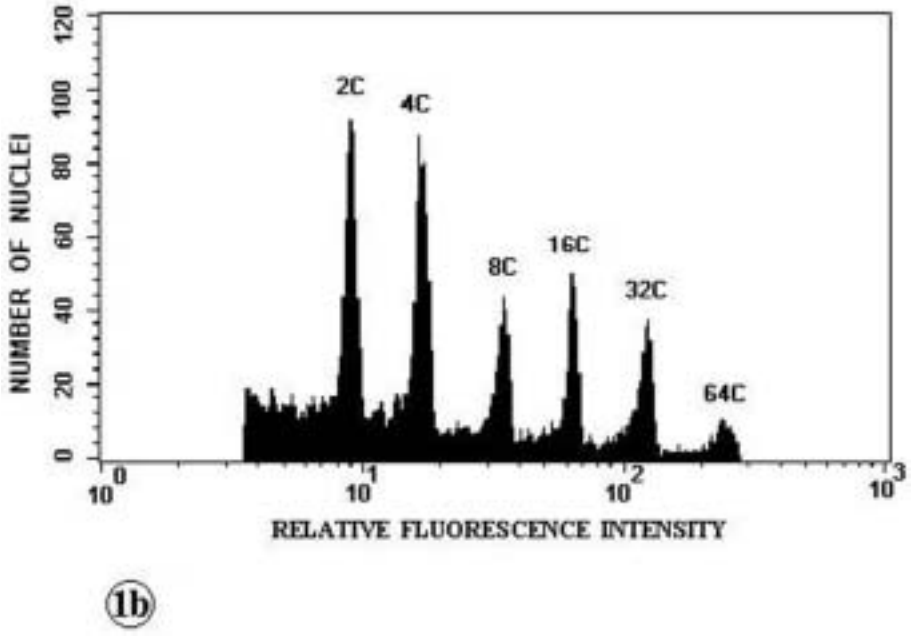
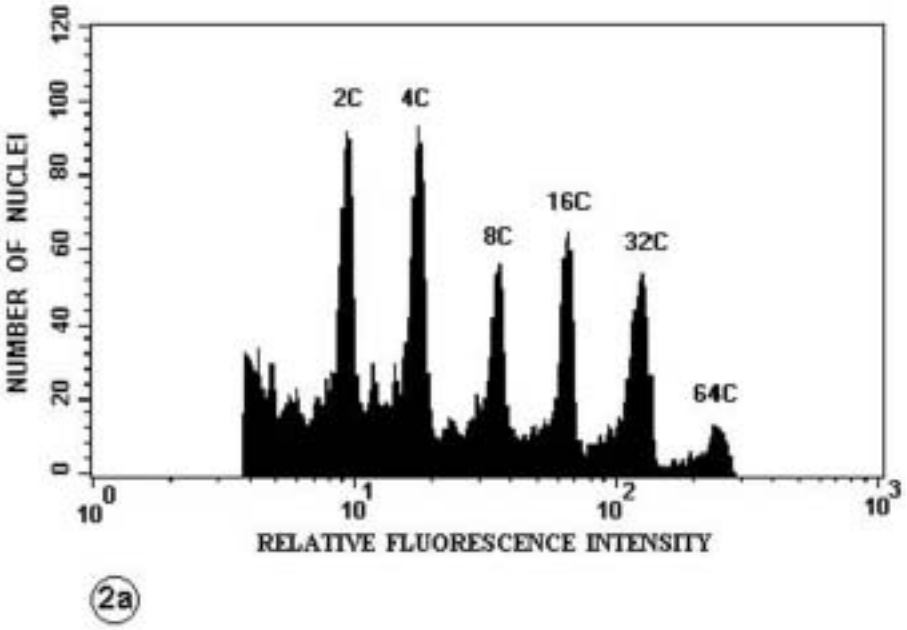


Fig. 1 — Histograms of relative DNA contents obtained after the analysis of DAPI-stained suspensions of nuclei prepared from two types of cotyledons, 'Cot' (A) and 'Cot È (B) of *Vigna radiata* Cv. B-1.

Data analysis - Analysis of relative fluorescence intensity of nuclei isolated from plant tissue free of endoreduplicated cells yields a histogram showing a major peak corresponding to the nuclei in the G1 phase of the cell cycle and a minor peak corresponding to G2 nuclei. To analyze ploidy level, the portion of the G1 peak on a histogram is compared to that of a reference tissue with known ploidy. In this investigation, isolated nuclei of *V. radiata* embryo were used as standard for 2C value and the frequencies of nuclei with different DNA C-levels were determined. All the experiments were repeated at least three times after standardization. Endopolyploidy analysis of FCM data was performed using Cell Quest software (Becton Dickinson).

RESULTS AND DISCUSSION

The present study enumerates that the DNA content of cells of *V. radiata* cotyledon varies from



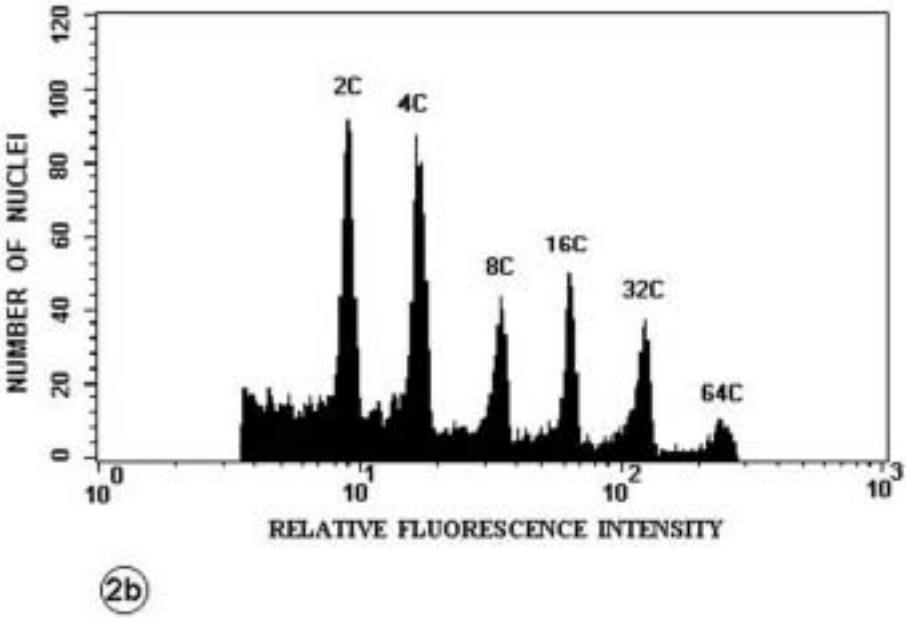


Fig. 2 — Histograms of relative DNA contents obtained after the analysis of DAPI stained suspensions of nuclei prepared from two types of cotyledons, 'Cot' (A) and 'Cot È (B) of *Vigna radiata* Cv. T-44.

2C to 64 C, where 2C is a relative DNA content of a diploid nucleus in G1 phase of the cell cycle (Table 1). The contribution of 2C nuclei is >22%, 4C nuclei >23%, 8C nuclei >13%, 16C nuclei >12%, 32C nuclei >8% and 64 C is either absent as in Cv. ML-2 or maximum 7.1% as in Cv. PIMS-4. In the cultivar ML- 2, only up to 32C DNA content was noted and a small amount of nuclei with 64C DNA content in the Cv. B-1 was observed (Fig. 1). The maximum amount (3.4%) of 64 C DNA was present in Cv. T-44 (Fig. 2). Whereas, the DNA content of maize endosperm varies from 3C to 96 C (DUNPHY and NEWPORT 1988), where contribution of cells containing 3C genome content is as low as 15%.

The difference in the distribution pattern of genomic DNA level was noted amongst the different cultivars (vide Table 1). This is the first report on the FCM analysis showing variation in the level of endoreduplication amongst the cotyledons of different *V. radiata* cultivars. However, there was no appreciable difference in the different C-levels of genomic DNA content between the two cotyledon types.

KNOWLES and PHILLIPS (1988) affirmed that endoreduplication happens to be an essential process for the development of *Zea mays* endosperm. However, significance of genome endoreduplication in cotyledons of grain legumes and endosperm of cereals are yet to be clarified (LARKINS *et al*. 2000). Presumably genomic endoreduplication in cotyledons of *V. radiata* provides the toughness to protect the dormant embryo from the environmental stress. Unravelling of the control of process regulating endoreduplication may focus the possible mechanism by which the productivity of storage tissue like cotyledons of legumes (SMITH 1981) and endosperms of maize (KNOWLES and PHILLIPS 1988; SCHWERZER *et al*. 1995) increases. Thus, analysis of DNA endoreduplication level in the cotyledonary tissue may provide the basis of selection for an increase in the storage tissues in all grain-legumes.

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