ORIGINAL ARTICLE

Flow cytometric analysis of oil palm: a preliminary analysis for cultivars and genomic DNA alteration

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Abstract

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DNA contents of oil palm (*Elaeis guineensis* Jacq.) cultivars were analyzed by flow cytometry using different external reference plant species. Analysis using corn (*Zea mays* line CE-777) as a reference plant gave the highest DNA content of oil palm (4.72±0.23 pg $2C^{-1}$) whereas the DNA content was found to be lower when using soybean (*Glycine max* cv. Polanka) (3.77±0.09 pg $2C^{-1}$) or tomato (*Lycopersicon esculentum* cv. Stupicke) (4.25±0.09 pg $2C^{-1}$) as a reference. The nuclear DNA contents of *Dura* (D109), *Pisifera* (P168) and *Tenera* (T38) cultivars were 3.46±0.04, 3.24±0.03 and 3.76±0.04 pg $2C^{-1}$ nuclei, respectively, using soybean as a reference. One haploid genome of oil palm therefore ranged from 1.56 to 1.81±10^9 base pairs. DNA contents from one-year-old calli and cell suspension of oil palm were found to be significantly different from those of seedlings. It thus should be noted that genomic DNA alteration occurred in these cultured tissues. We therefore confirm that flow cytometric analysis could verify cultivars, DNA content and genomic DNA alteration of oil palm using soybean as an external reference standard.

Key words : DNA content, *Elaeis guineensis*, flow cytometry, oil palm

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การวิเคราะห์ปาล์มน้ำมันโดยใช้โฟลไซโทเมทรี : การจำแนกเบื้องต้นสำหรับสายพันธุ์และการเปลี่ยนแปลงของจีโนมดีเอ็นเอ

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วิเคราะห์ปริมาณดีเอ็นเอของปาล์มน้ำมัน (*Elaeis guineensis* Jacq.) ด้วยวิธีโฟลไซโทเมทรี โดยใช้พืชอ้างอิงที่แตกต่างกัน โดยวิเคราะห์แยก พบว่าการใช้ข้าวโพด (*Zea may* line CE-777) เป็นพืชอ้างอิง ให้ปริมาณดีเอ็นเอของปาล์มน้ำมันสูงสุด (4.72±0.23 พิโคแกรม) ขณะที่ปริมาณดีเอ็นเอของปาล์มน้ำมันมีค่า 3.77±0.09 และ 4.25±0.09 พิโคแกรม เมื่อใช้ถั่วเหลือง (*Glycine max* cv. Polanka) และมะเขือเทศ (*Lycopersicon esculentum* cv. Stupicke) เป็นพืชอ้างอิง ตามลำดับ ปริมาณดีเอ็นเอของปาล์มน้ำมันสายพันธุ์ดูร่า พิสิเฟอร่า และเทเนอร่า มีค่าเท่ากับ 3.46±0.04, 3.24±0.03 และ 3.76±0.04 พิโคแกรม ตามลำดับ โดยใช้ถั่วเหลืองเป็นพืชอ้างอิง ดังนั้นระดับจีโนมที่เป็นแฮพลอยด์ของปาล์มน้ำมันจึงมีค่าอยู่ในช่วง 1.56 ถึง 1.81±10[9] คู่เบส ปริมาณดีเอ็นเอของแคลลัสและเซลล์แขวนลอยปาล์มน้ำมันที่เลี้ยงในสภาวะปลอดเชื้อมาเป็นเวลาหนึ่งปี มีความแตกต่างกันอย่างมีนัยสำคัญกับต้นกล้าที่เลี้ยงในสภาวะปลอดเชื้อ แสดงให้เห็นถึงการเปลี่ยนแปลงปริมาณดีเอ็นเอระหว่างแคลลัสและเซลล์แขวนลอย ผลที่ได้ยืนยันการตรวจสอบสายพันธุ์ ปริมาณดีเอ็นเอ และการเปลี่ยนแปลงระดับดีเอ็นเอของปาล์มน้ำมันด้วยวิธีโฟลไซโทเมทรี โดยใช้ถั่วเหลืองเป็นพืชอ้างอิงชนิดวิเคราะห์แยก

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Flow cytometry has been applied to estimate nuclear DNA content in several plant species (Bennett and Leitch, 1995; Dolezel *et al*., 1998). It offers a simple, rapid, accurate and convenient method for determining ploidy levels of DNA, assessment and analysis of the cell cycle of large cell populations (Winkelmann *et al*., 1998; Dolezel, 1991). However, the choice and correct use of reference standards is a criterion which has been largely neglected (Dolezel *et al*., 1998). For oil palm, discrepancies are quite large and might be attributable not only to the differences among the techniques, but also to the type of standard used for estimation (Rival *et al*., 1997). Therefore, the potential effects in each reference plant on DNA content of oil palm need to be investigated more extensively.

It is generally known that a database of ploidy level and DNA content is important in any breeding program of new plant cultivars. Oil palm, based on the fruit structure, has been classified as *Dura* (thick shell; less mesocarp), *Pisifera* (shell-less; embryo rarely formed) and the commercially cultivated *Tenera*, the D×P hybrid (thin shell; more mesocarp: 60-95%), with high oil content. *Elaeis guineensis* Jacq. cv. *Tenera* is a valuable economically important source of vegetable oil, the most traded vegetable oil in the international market, and is increasingly used in the food industry. DNA content of oil palm is rather variable, especially due to the high potential of conventional cross breeding among *Dura* and *Pisifera* cultivars in the first filial hybrid trait. Although DNA content of *Elaeis* cv. *Tenera* was reported previously for 3.7 pg (Rival *et al*., 1997), flow cytometric studies of the nuclear DNA content in their parents, *Elaeis* cv. *Dura* and *Pisifera*, have not been described to date.

At present, clonal propagation of oil palm through tissue culture is common (de Touchet *et*

al., 1991; Teixeira *et al*., 1995; Aberlenc-Bertossi, 1999; Rajesh *et al*., 2003) and plant regeneration is mostly achieved through callus or cell suspension cultures. Genome size of these cultured tissues can be altered through changes either in the chromosome number or in the ploidy level, namely somaclonal variation. In this study, we investigated these phenomena by examining DNA content of different *in vitro* cultured tissues i.e. calli, cell suspension and shoot of seedlings using soybean as an external reference plant. Although there are many potential markers to investigate abnormality of oil palm, difficulties have been encountered when using these procedures on a large scale (Rival *et al*., 1997). Our attention was focused on the investigation of oil palm DNA content determined by flow cytometry using different reference plant species as an external reference. Moreover, DNA contents of their parents and long-term *in vitro* cultured tissues were clearly defined.

Materials and Methods

Plant material

E. guineensis Jacq. cv. *Dura, Pisifera* and *Tenera* used in this study were prepared at Surat Thani Oil Palm Research Center, Surat Thani Province, Thailand. The following reference seeds (soybean, *Glycine max* cv. Polanka; tomato, *Lycopersicon esculentum* cv. Stupicke and corn, *Zea mays* line CE-777) were kindly provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic. All seeds were sown in pots and plants were grown in a greenhouse prior to use for sample preparation.

One-year-old calli and cell suspension at the 3rd subculture and seedlings were prepared prior to analysis by flow cytometry. Calli were maintained on MS (Murashige and Skoog, 1962) medium containing 4.1-41.4 µM picloram or 4.5-45.2 µM dicamba. MS liquid medium supplemented with 5.3 µM NAA (α-napthaleneacetic acid) and 4.5 µM 2,4-D (2,4-dichlorophenoxyacetic acid) and the medium without growth regulator were used for cell suspension and seedling culture. All cultured tissues were transferred to a new medium at 4-month intervals.

Flow cytometric analysis

Soybean, tomato and corn were used as external reference plants (2C=2.5; Afza *et al*., 1993: 2C=1.96; Dolezel *et al*., 1992 and 2C=5.72; Dolezel *et al*., 1992, respectively). Young leaves of oil palm cultivar *Dura* and *Pisifera* and embryonic tissues of *Tenera* and the 2nd - 3rd leaves from shoot apex of reference plants, approximately 20-30 mg, were finely chopped with a razor blade in a 1.0 ml Tris-$MgCl_2$ extraction buffer containing 0.2 M Tris, 4 mM $MgCl_2$, 0.5%(w/v) Triton X-100 and 3.0%(w/v) polyvinylpyrrolidone (PVP). After extraction, 50 µl of RNase and propidium iodide (PI) were added immediately prior to filtering through 42 µm nylon mesh (Pfosser *et al*., 1995). One-year-old tissues of calli, cell suspension and shoot meristem of seedlings were also prepared as above using soybean as an external reference. The nuclear DNA content (in pg) of oil palm samples was estimated according to the equation: 2C nuclear DNA content = (reference in pg×2C peak mean of oil palm)/(2C peak mean of reference). The number of base pairs per haploid genome (Bp $1C^{-1}$ nuclei) was calculated based on the equivalent of 1 pg DNA=965 Mega basepair (Bennett and Smith 1976).

The flow cytometer was a FACScalibur (Becton Dickinson Biosciences, San Jose, CA) working with the software CellQuest (Becton Dickinson) equipped with 488 nm argon iron laser. PI was measured at 585 nm to read 2C nuclei DNA contents of 5,000 nuclei per sample. During analysis, after every three samples, the reference plant was controlled to check the calibration of the flow cytometer by adjusting the gain of *Glycine* to channel 200. All experiments were carried out at least 3 times with 5-10 replicates per treatment.

Data analysis

The fluorescence histograms were resolved into G0/G1 (2C), S and G2/M (4C) cell-cycle compartments with a peak-reflect algorithm using two Gaussian curves (WinMDI version 2.8). DNA

contents of oil palm were statistically analyzed by ANOVA (Analysis of variance) and the significant difference between the contents was tested against the *F*-distribution at $p \leq 0.05$. Tukey test was performed for routine multiple mean comparison.

Results

Effect of different reference plants used on DNA content of oil palm

DNA contents of oil palm cv. *Tenera* were analyzed using soybean (2C=2.5; Afza *et al*., 1993), tomato (2C=1.96; Dolezel *et al*., 1992) and corn (2C=5.72; Dolezel *et al*., 1992) as external reference plants. DNA contents of oil palm were 3.77±0.18, 4.25±0.18 and 4.72±0.46 pg $2C^{-1}$ nuclei when soybean, tomato and corn were used as the reference plant, respectively. Thus base pairs of one haploid genome ranged from 1.82×10^9 bp to 2.28×10^9 bp (Table 1). Although soybean did not produce the highest estimate of DNA content of oil palm, this DNA content of oil palm (3.77±0.18 pg $2C^{-1}$) (Figure 1) most closely agreed with Rival *et al*. (1997) who analyzed DNA content of oil palm on FACScan cytometer using petunia (*Petunia hybrida*) as an internal reference plant. We therefore used soybean as an external reference plant for routine DNA content analysis of oil palm on FACScalibur cytometer.

DNA content of *Dura, Pisifera* and *Tenera*

Analysis of three cultivars of oil palm, namely *Dura* (D109), *Pisifera* (P168) and their crossed F1 hybrid, *Tenera* (T38), revealed significant differences ($p \leq 0.0001$). *Tenera* (T38) gave the highest DNA content (3.76±0.04 pg $2C^{-1}$) while DNA contents of its parents, *Dura* (D109) and *Pisifera* (P168), were lower (3.46±0.04 and 3.24± 0.03 pg $2C^{-1}$, respectively) (Figure 2 and Table 2). Therefore, the numbers of base pairs of one haploid genome were 1.67, 1.56 and 1.81×10^9 bp for *Dura, Pisifera* and *Tenera*, respectively.

Investigation of genomic DNA alteration

To investigate altered DNA content, somaclonal variation, of *in vitro* oil palm cultured for one year, *Glycine* was used as an external reference plant. DNA contents of cultured oil palm tissues obtained in our experiments are listed in Table 3. The results revealed that one-year-old calli, cell suspension and shoot meristem of seedling at the third subculture on MS medium containing 4.1-41.4 µM picloram or 4.5-45.2 µM dicamba, in liquid MS medium supplemented with 5.3 µM NAA and 4.5 µM 2,4-D and in liquid MS medium without growth regulator were significantly different in their DNA contents (4.17±0.25, 4.07±0.03 and 3.77±0.01 pg $2C^{-1}$, respectively, p 0.0001) (Figure 3 and Table 3).

Discussion

When the three species of external reference plants (soybean, tomato and corn) were used to

Table 1. Genome size (2C nuclear DNA content in pg) of *E. guineensis* Jacq. compared with various reference plants. The means are based on 10 replicated experiments.

Reference plants	DNA content (pg $2C^{-1}$) (Mean±SE)*		
	Reference plants	*Elaeis* (external reference)*	*Elaeis* (Base pairs)
G. max cv. Polanka	2.5[2]	3.77±0.09[c]	1.82×10^9
L. esculentum cv. Stupicke	1.96[1]	4.25±0.09[b]	2.05×10^9
Z. mays line CE-777	5.72[1]	4.72±0.23[a]	2.28×10^9

[1]Dolezel *et al.* (1992); [2]Afza *et al.* (1993)
* Values not having superscript letters in common are significantly different at the 0.05 probability level by Tukey test.

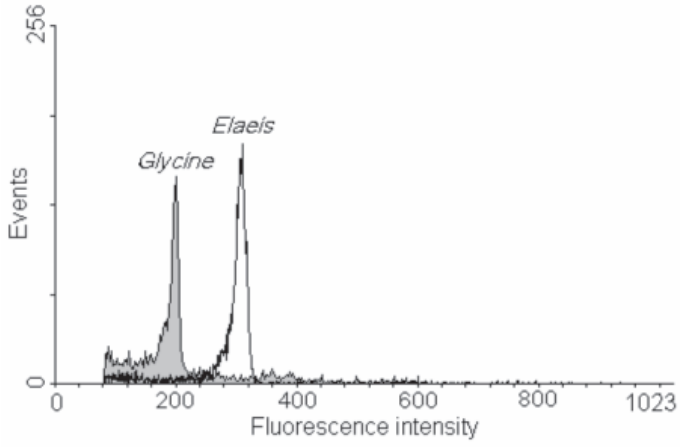

Figure 1. Histogram of fluorescence intensity of nuclei isolated from oil palm using soybean (*Glycine*) as an external reference plant.

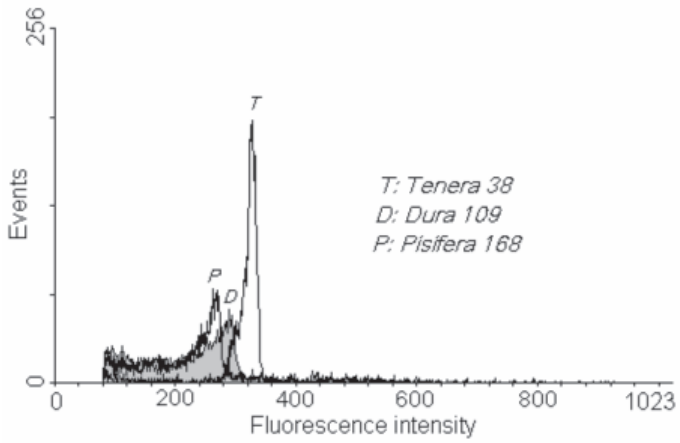

Figure 2. Comparison of fluorescence intensity histogram among *E. guineensis* Jacq. cv. *Dura* (D109), *Pisifera* (P168) and *Tenera* (T38) using soybean (*Glycine*) as an external reference plant.

Table 2. Genome size (2C nuclear DNA content in pg) of *E. guineensis* Jacq. cv. *Dura, Pisifera* and *Tenera* using *Glycine* as an external reference standard. The means are based on 5 replicated experiments.

Elaeis cultivars	DNA content (pg $2C^{-1}$) (Mean±SE)*	*Elaeis* (Base pairs)
G. max cv. Polanka (external reference)	2.5[1]	1.21×10^9
Dura (D109)	3.46±0.02[b]	1.67×10^9
Pisifera (P168)	3.24±0.01[c]	1.56×10^9
Tenera (T38)	3.76±0.02[a]	1.81×10^9

[1]**Afza *et al.* (1993)**

*** Values not having superscript letters in common are significantly different at the 0.05 probability level by Tukey test.**

Table 3. Genome size (2C nuclear DNA content in pg) of one-year-old of calli, cell suspension and shoot meristem of seedlings at the 3rd *in vitro* subculture. The means are based on 5 replicated experiments.

Explants of oil palm	DNA content (pg $2C^{-1}$) (Mean±SE)*
G. max cv. Polanka (external reference)	2.5[1]
Shoot meristem	3.77±0.01[c]
Calli	4.17±0.05[b]
Cell suspension	4.27±0.03[a]

[1]**Afza *et al.* (1993)**
***Values not having superscript letters in common are significantly different at the 0.05 probability level by Tukey test.**

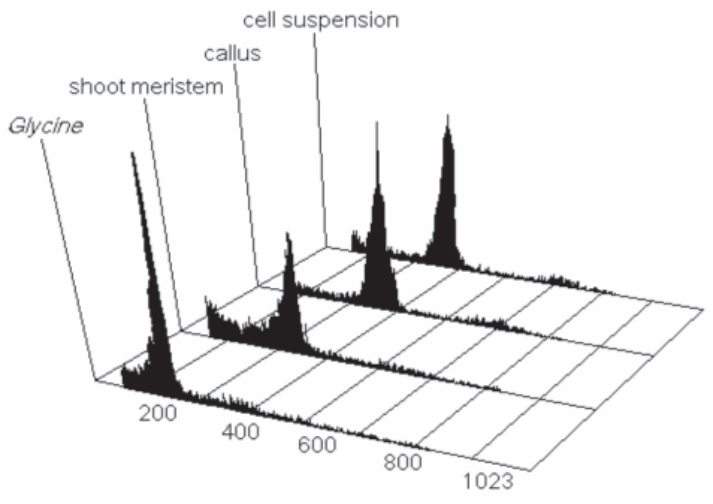

Figure 3. Comparison of fluorescence intensity histogram of oil palm among *in vitro* cultured tissues of shoot meristem, calli and cell suspension using soybean (*Glycine*) as a reference plant.

verify the DNA content of oil palm, the results revealed significant differences in DNA content among the references used. The highest DNA content of oil palm (4.72 pg $2C^{-1}$) was found when using corn as an external reference whereas using soybean as a reference plant gave the lowest DNA content (3.77 pg $2C^{-1}$). Soybean has been described as a reference for banana (Lysak *et al*., 1999) but tomato and corn have been little used. In Arecaceae, *Petunia hybrida* (2C=2.85) was mostly used as a reference in flow cytometric analysis such as that of date palm (Siljak-Yakovlev *et al*., 1996), oil palm (Rival *et al*., 1997) and coconut (Sandoval *et al*., 2003); however, we could not get petunia for this investigation. Although the technique and equipment used here were different, it should be noted that our results of oil palm DNA content using soybean as an external reference most resembled those of Rival *et al*. (1997). When the references were used as an internal standard, the results showed that soybean gave the different reading in peak position of oil palm from tomato and corn (unpublished data), resulting from interference between the staining solution and the

genome of the two species (Amsellem *et al.,* 2001). Although 2C peak of oil palm did not overlap with those references, the 2C peak of corn gave poor reading in peak quality whereas 2C peak of soybean was located closer to that of oil palm than tomato, which was helpful in decreasing the error of DNA content. We thus showed that soybean could be used as an external reference plant for DNA content analysis of oil palm.

For cultivar investigation, we used young leaves of *Dura* and *Pisifera* collected from adult oil palm (non-opened leaves). Because we could not obtain the seeds from self-fertilization, only Tenera embryos, their F1 hybrid, were investigated simultaneously. Unfortunately, only one cross of oil palm was used in this study since a single oil palm cv. *Tenera* (T38) was used in our experiments for *in vitro* cultured previously. Thus the DNA content of *Dura* (D109) and *Pisifera* (P168) cultivars, female and male parent plants of *Tenera* (T38), was also investigated and compared to that of their hybrid. It is interesting to note that DNA content of *Tenera* reported here was found to be clearly significantly higher than that of their parents indicating that it might carry specific DNA from both parents, thereby confirming their hybridity (Hirsch *et al*.,2001). These DNA contents are firstly reported on *Dura* and *Pisifera* cultivars and can be used as a database in any further oil palm breeding programs and may be helpful in verifying cultivars or clone. Although many interesting agronomic characters have been found in *Tenera* cultivar, truly a large variability in quantitative traits is found in this cultivar.

Although our results confirm those obtained by Rival *et al.* (1997), DNA contents of cultured tissues were different. DNA contents of calli and cell suspension reported here were significantly higher than that of shoot meristem of seedling (Table 3) whereas Rival *et al.* (1997) reported that DNA contents of cultured tissues were lower than that of plantlets. The occurrence of endopolyploidy in differentiated plant tissues is well known and it is not characterized as an original feature (Kudo and Kimura, 2001) and nuclei distribution depends on types of tissues cultured *in vitro* (Sandoval *et al*., 2003). Our results thus assumed that endopolyploidy might occur in calli and cell suspension of oil palm in long-term culture and might have been affected by plant growth regulators and the stress conditions of the culture.

In conclusion, we have the possibility to carry out DNA content of oil palm on FACScalibur flow cytometer using soybean as an external reference plant. The DNA content of oil palm crossed by economic important cultivars mostly a useful database for routine breeding program and for routine assay of an accepted cultivated clone. In addition, the results from flow cytometry can be a good indicator of cell division activity in the cell cycle or of the potential of tissues for culture *in vitro* (Winkelman *et al*., 1998; Sandoval *et al*., 2003; Srisawat and Kanchanapoom, 2005).

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