3. METERIALS AND METHODS

3.1 Seedling and callus induction

3.1.1 Plant materials

Oil palm mature seeds, cultivar *Tenera*, were kindly provided by Suratthani Oil palm Research Center, Suratthani Province, Thailand. The epicarp and pulp were removed prior to removing the endocarp then the kernels embedded zygotic embryos were first surface-disinfected with 70% ethanol for 1 min, then soaked in 40% (v/v) commercial Clorox™ solution containing 0.5 ml of Tween-20 emulsifier per 100-ml solution for 1 h and then rinsed at least three times with autoclaved distilled water. The kernels containing embryos were aseptically immersed in sterilized distilled water for 0, 24, 48 or 72 h. Intact embryos were excised from the kernels with a scalpel filled with a #11 blade and transferred to culture medium.

3.1.2 Seedling and callus initiation

Excised embryos were initially cultured on either liquid or solid Y3 medium (Eeuwens, 1976) without plant growth regulators for seedling germination. The liquid Y3 media supplemented with 4.1-8.3 μM picloram (4-amino-3,5,6-trichloropicolinic acid), 4.5-13.6 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 0.56 mM myo-inositol and 131 mM sucrose were used for callus induction. Callus initiated from embryos was observed for 4 weeks.

3.1.3 Culture media and conditions

The pH of all media was adjusted to 5.7 prior to the addition of 0.82% Pearl Mermaid™ commercial gelling agent and autoclaved at 121°C, 1.2-1.3 kg cm^{-2} pressure for 20 min. All cultures were incubated at 25 ± 2°C air temperatures in a culture room with a 16h photoperiod at 20 μmol $m^{-2}s^{-1}$ photosynthetic photon fluxes at plant level provided by cool-white fluorescence. Five embryos were planted per culture vessel. All experiments were carried out at least three times with five cultures per treatment.

3.2 Flow cytometric analysis

3.2.1 Plant materials

The following reference seeds (*Glycine max* cv. Polanka, *Lycopersicon esculentum* cv. Stupicke and *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.

For genomic DNA alteration, one-year-old calli 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 (3,6-dichloro-o-anisic acid). MS liquid medium without growth regulators was used for seedling maintenance. All cultured tissues were transferred to a new medium at 4-month intervals.

Oil palm embryos imbibed for 24-72 h, seedlings and calli from 3.1.2 were used as plant materials for cell cycle activity comparison.

3.2.2 Reference plants testing

Glycine max cv. Polanka, *Lycopersicon esculentum* cv. Stupicke and *Zea mays* line CE-777 were used as internal 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). Oil palm tissues, approximately 20-30 mg, were finely chopped with a razor blade in a 1 ml Tris-$MgCl_2$ extraction buffer containing 0.2 M Tris, 4 mM $MgCl_2$, 0.5%(w/v) Triton X-100 and 3%(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).

3.2.3 Genomic DNA alteration

One-year-old tissues of calli and shoot meristem of seedlings were also prepared as above using *Glycine max* cv. Polanka as an internal reference.

3.2.4 Cell cycle activity analysis

The proportions of cells in the G2 phase of the cell cycle in embryos imbibed for 24-72 h, calli and seedling were compared (in percentage).

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 max* cv. Polanka to channel 200. All experiments were carried out at least 3 times with 5-10 replicates per treatment.

3.2.5 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). The DNA content and the number of cells in the G2 phase in each explant of oil palm were statistically analyzed by ANOVA (analysis of variance) at $p \leq 0.05$. Tukey test was performed for routine multiple mean comparison.

3.3 Protoplast isolation and culture

3.3.1 Plant materials

Mature seeds of oil palm (*Elaeis guineensis* Jacq.) cv. *Tenera* were kindly provided by Suratthani Oil palm Research Center, Suratthani Province, Thailand. Surface-sterilized kernels containing embryos were aseptically immersed in sterile

distilled water for 48 h and used for isolating protoplasts. Calli, basal leaves and shoots of seedlings from 3.1.2 were also used as protoplast sources.

3.3.2 Protoplast isolation

Calli, basal leaves, shoots of seedlings and imbibed-embryos were preplasmolysed in 5 ml of CPW (Power *et al.*, 1984) salt solution with 0.7 M sorbitol for 1 h in the dark. After removing CPW solution, they were chopped transversely about 1-2 mm in width and then treated with the enzyme solutions. The enzyme solutions contained Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd. Lot # 201059), Driselase (Kyowa Hakko Co., Ltd. Lot # 4111) and Macerozyme R-10 (Yakult Honsha Co., Ltd. Lot # 202021) in CPW salts with 0.6 M sorbitol at pH 5.7 unless otherwise stated. Five ml of filter-sterilized (Millipore™ filter, 0.45 μm pore size) enzyme solution in 60-mm Corning Petri dishes were used for 0.2 g of tissues. Protoplast sources were separated into three groups depending on the mixture of enzymes used. The sliced calli, basal leaves and shoots of seedlings were incubated in the enzyme solutions containing 3% Cellulase Onozuka R-10, 1-2.5% Driselase and 1% Macerozyme, on a rotary shaker at 30 rpm for 3 h in darkness. The slices of friable calli cultured in liquid medium (from 3.1.2) were incubated in the enzyme solution containing 1% Cellulase Onozuka R-10, 0.5% Driselase and 1% Macerozyme R-10, on a rotary shaker at 30 rpm for 20-48 h in darkness. Lastly, the sliced 48h-imbibed embryos were incubated in the enzyme solutions containing 1% Cellulase Onozuka R-10, 0.15-0.5% Driselase and 0.25-0.5% Macerozyme R-10, without agitation for 24-72 h in darkness.

3.3.3 Protoplast purification

For protoplast purification, after incubation, enzyme-protoplast mixtures were gently filtered successively through a 140 μm stainless steel sieve and 77 and 42 μm nylon mesh to remove any clumps of undigested tissues and debris, and collected into a 15-ml screw capped centrifuge tube. The protoplast suspension was pelleted by centrifugation at 52×g for 8 min. The supernatant was removed and the pelleted protoplasts were resuspended in 0.6 M sucrose. Sorbitol solution of 0.6 M was overlaid on the top of this sucrose solution and the tube was centrifuged at 52×g for 10 min. A thick band of protoplasts was formed in the interface between sucrose and sorbitol layers while the remaining cells and debris were sedimented to the bottom of the tube. Protoplasts from this band were withdrawn gently by Pasteur pipette and washed 2-3 times with washing medium in 0.5 M sorbitol.

3.3.4 Yield and viability measurements

Purified protoplast samples were collected from each treatment and counted using an AO Bright-Line hemacytometer slide at 100× magnification. The viability of protoplasts was monitored using fluorescein diacetate (FDA) (Sigma) using the procedure as described by Widholm (1972). The presence of wall material was monitored using Calcofluor white (Sigma) as described by Nagata and Takebe (1970). The fluorescence of the viable protoplasts was determined using an Olympus inverted microscope model IMT-2 using a blue excitation filter (490-515nm) and wall formation using ultraviolet excitation filter (365-420nm) combination.

3.3.5 Protoplast culture methods and protoplast sources investigation

Washed protoplasts from basal leaves, calli and imbibed embryos were plated at 1×10^5 protoplasts per ml and cultured by agarose bead (0.4% Agarose Type VII, Sigma, USA), 0.4% Agarose overlaid with liquid thin layer and semi-solid medium (0.3% Agarose) techniques. Agarose bead was surrounded by liquid medium with the same composition and renewed at 2-week intervals. All media contained MS medium salts supplemented with 8.3 μM picloram, 4.5 μM 2,4-D, 8.8 μM BA (N^6-benzyladenine) and 4.6 μM kinetin (6-furfurylaminopurine).

3.3.6 Gelling agents and carbon sources investigation

The density of protoplasts isolated from imbibed embryos was adjusted to 1×10^5 protoplasts per ml in 2 ml beads of MS medium containing various types and concentrations of gelling agents [0.4% agarose Type VII (Sigma, USA), 0.3% Gelrite (Merck & Co. Inc., USA) and 1.8% sodium alginate (Wako, Pure Chemical Industries, Japan)], carbon sources (0.4 M glucose, 0.38 M glucose plus 0.02 M sorbitol and 0.38 M glucose plus 0.02 M mannitol) and plant growth regulators (13.6 μM 2,4-D, 4.4 μM BA and 9.3 μM kinetin). Protoplast cultures were placed in the dark at a constant temperature of 25±2 °C. Percentage of cell division was calculated after 2 weeks of culture.

3.3.7 Cell division induction

Media containing 4.1-12.4 μM picloram, 4.5-18.1 μM 2,4-D, 9.3 μM kinetin and 4.4 μM BA and 0.1% activated charcoal in a 60-mm Corning Petri dish were

used for increasing cell division using the technique of agarose beads containing glucose plus sorbitol as carbon source. Two ml of MS liquid medium containing the same plant growth regulators were added to surround the beads. Protoplast cultures were placed in the dark at a constant temperature of 25±2°C. Percentage of cell division was calculated after 2 weeks of culture.

3.3.8 Cell colony formation

When cell division was evident, the osmoticum concentration of the medium was gradually reduced at 14-day intervals by replacing the liquid medium around the beads with the fresh medium with the osmoticum concentration reduced to half, 0.1% activated charcoal and different combinations of the following plant growth regulators, i.e., picloram (0, 4.1 μM), 2,4-D (0, 4.5 μM), dicamba (0, 4.5 μM), kinetin (9.3 μM), BA (4.4 μM) and ABA (abscisic acid) (3.8 μM). Plating efficiency was calculated at the 4th week of culture.

3.3.9 Data analysis

The yield of protoplasts and the percentage of dividing cells were obtained from two independent experiments with three replicates per isolation and culture treatments. The frequency of cell division was calculated after 2 weeks of culture whereas the percentage of cell colony formation in terms of planting efficiency (PE) was calculated at 4th week of culture. Four to five protoplast samples were taken randomly from each treatment for the determination of their means and standard errors and the data were analyzed by ANOVA (analysis of variance) in which $p \leq 0.05$ was considered to be statistically significant.