African Journal of Biotechnology Vol. 11(82), pp. 14761-14770, 11 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1351
ISSN 1684–5315 ©2012 Academic Journals

*Full Length Research Paper*

# Recloning of regenerated plantlets from elite oil palm (*Elaeis guineensis* Jacq.) cv. Tenera

**Mya Thuzar[1], Apichart Vanavichit[2], Somvong Tragoonrung[3] and Chatchawan Jantasuriyarat[4,5]***

[1]Tropical Agriculture Program, Department of Agronomy, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
[2]Rice Science Center and Department of Agronomy, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
[3]Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), Klong Luang, Pathum Thani 12120, Thailand.
[4]Department of Genetics, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.
[5]Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University (CASTNAR, NRU-KU), Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

Accepted 23 July, 2012

**Plant regeneration in oil palm cv. Tenera via somatic embryogenesis was conducted using regenerated plantlets as an explant source. Explants from different positions – apex, middle and basal segments of regenerated plantlets – were cultured in N6 medium supplemented with 100, 120 and 140 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of activated charcoal. The production of embryogenic calli was affected by 2,4-D concentration and explant position; 2,4-D 120 mg/L was the most effective (62.53%) in inducing embryogenic calli from the basal segment 5 months after inoculation. After 3 months of culture in embryo maturation medium, an average of 36 ± 8 somatic embryos per embryogenic callus was obtained. When transferred to plant regeneration medium for 3 to 4 months, these somatic embryos differentiated into shoots, with ranges of 6 to 40 and 4 to 32 shoots on the medium with and without 2-isopentyladenine (6-dimethylaminopurine) (2iP), respectively. Plantlets (6 to 8 cm height) with balanced shoots and roots were obtained after 12 to 14 months. Histological analysis confirmed the initiation, development and germination of somatic embryos from explants of regenerated plantlets. Simple sequence repeat (SSR) analysis showed the genetic identity and uniformity between the first and second regenerated plantlets at five SSR loci.**

**Key words:** *Elaeis guineensis* Jacq., genetic identity, regenerated plantlets, somatic embryogenesis, SSR marker.

## INTRODUCTION

As a major source of edible oil in the world, the oil palm (*Elaeis guineensis* Jacq.) is a key resource, especially in the vegetable oil and biofuel industries. Oil palm is a perennial and open-pollinated plant, whose breeding cycle usually takes more than ten years. In nature, there are three varietal types of oil palm which have been classified on the basis of the presence or absence of a shell in the fruit: Dura which produces fruits with a thick shell; Pisifera which is without a shell in its rare fruits; Tenera which is a hybrid of these two variety types and produces fruits with an intermediate shell. The Tenera varieties, which are derived from Dura x Pisifera crosses and Dura x Tenera crosses, are naturally more productive in oil. The most commercial oil palms are high yielding Tenera varieties; however, this cultutivar typically

*Corresponding author. E-mail: fscicwj@ku.ac.th. Tel: +66 8 3019 1157. Fax: +66 2579 5528.

shows considerable variation in oil yield and other agronomically important traits. Most importantly, in Thailand, it is difficult to obtain a required amount of high yielding, uniform hybrid 'Tenera' palms for large scale cultivation. Therefore, the development of an efficient plant regeneration system for oil palms is of vital importance for clonal propagation of elite 'Tenera' variety. Oil palm clones offer the potential for higher yield/ productivity because it is possible to establish uniform trees comprised of identical clones of highly productive genotypes.

Although many research works on oil palm tissue culture have been conducted since 1970 using different types of explant tissues, it is still difficult to obtain embryogenic callus from certain genotypes (Rival and Parveez, 2004). In palms, somatic embryogenesis (SE) is a promising regenerative route, as this morphogenetic pathway may increase the number of regenerated plantlets in comparison with organogenesis (Steinmacher et al., 2007). Somatic embryogenesis is the developmental process by which somatic cells develop into structures that resemble zygotic embryos through an orderly series of characteristic embryological stages without fusion of gametes (Jiménez et al., 2001). This developmental route can be induced by many factors such as explants, media, hormones, and culture condition (Fehér et al., 2003). Among the different auxin analogues, 2,4-D has been the most commonly applied for SE induction. In more than 65% of 124 recent protocols, 2,4-D was applied alone or in combination with other plant growth regulators (Gaj, 2004). Moreover, the developmental stages of explant tissues are considered to be one of the most important elements in acquiring embryogenic competence in palms (Steinmacher et al., 2007; Gueye et al., 2009; Scherwinski-Pereira et al., 2010).

Genetic conformity in plants propagated *in vitro* should be assessed at a very early stage of development, especially in perennial trees such as oil palm, which would reduce time, cost and resources required for the variety improvement. Microsatellite SSRs (simple sequence repeats) are one of the most informative markers due to their abundance, hypervariability and random distribution in eukaryotic genomes (Wang et al., 2005). The highly polymorphic nature of SSRs is of particular importance for oil palm, as the breeding palms often involve narrow gene pools. Additionally, it was demonstrated that the SSR markers can be used for clonal identification, monitoring line uniformity between and within clones and detecting culture mix-up in oil palm (Singh et al., 2007).

We aimed to examine the effects of 2,4-D concentration and explant position on the acquisition of embryogenic competence, using different tissue segments from regenerated plantlets, as explants. To validate the clonal fidelity of regenerated plantlets from two successive regenerations, genotypic screening of 1st and 2nd regenerated plantlets was examined using five SSR markers. This is a simple but useful technique that can be applied for using regenerated plantlets as a potential source of explants in oil palm micropropagation. Additionally, most commercially grown hybrid 'Tenera' typically shows high variation in oil yield and other agronomically important traits. If the desired genotype of hybrids at the seedling stage could be selected, after verifing their genetic component with the aid of molecular markers, *in vitro* cloning of elite seedling palms would be of great benefit to growers. This work can provide an efficient regeneration system for cloning / recloning of selected genotypes and also for multiplying promising seedlings such as the popular commercially grown cv. Tenera.

## MATERIALS AND METHODS

*In vitro* plantlets (about 7 to 8 cm in height) regenerated from cultured zygotic embryos of oil palm cv. Tenera were used in this study, based on the previous work of Thuzar et al. (2011). Plant samples obtained from different zygotic embryos were randomly selected. The leaves, roots and the outermost leaf sheath of plantlets were removed. Leaf samples of individual plantlets were kept at -80°C for SSR analysis. The remaining leaf tissue was cut into three segments, each 0.5 cm in length, starting from the base of the plant: basal, middle and apex segments. The basal segment was composed of apical meristem, leaf primordial and some young leaf tissues, while the other two segments were composed only of young leaf tissues. Afterwards, each segment was longitudinally cut in half; thus a total of six leaf tissue sections were obtained from each plantlet (Figure 1a). To evaluate the effect of explant position on production of embryogenic callus, six tissue sections from each plantlet (two parts from each of the three segments of leaf tissue) were inoculated in one Petri dish, with the longitudinal cutting edges facing the media. The explants were evaluated as basal, middle and apex segments.

### Culture media and conditions

The explants were inoculated on N6 basal medium (Chu et al., 1975) with 30 g/L sucrose, 3 g/L activated charcoal (AC) and 3 g/L Phytagel®. Our previous result showed that 2,4-D gave the highest embryogenic callus induction, which was significantly different from other auxins: picloram and dicamba. Therefore, in this experiment in the presence of 3 g/L AC in callus induction medium, 2,4-D was tested at different concentrations (100, 120 and 140 mg/L) to evaluate the most effective concentration on the production of embryogenic callus. Callus induction and embryogenic callus induction were evaluated at 3 and 5 months after inoculation, respectively. Subculture was applied every 4 weeks throughout the culture process. Embryogenic calli at the globular stage were transferred to a somatic embryo maturation medium composed of basal medium supplemented with 0.16 g/L putrescine, 0.5 g/L casein amino acids, and 0.04 g/L adenine sulfate. These cultures were maintained until somatic embryos developed into the cotyledonary stage. In order to regenerate the plantlets, the cotyledonary-stage somatic embryos were transferred to plant regeneration medium: modified N6 + 1 g/L AC) with or without 2 mg/L 2iP [2-isopentyladenine, 6-dimethylaminopurine]. Modified N6 is the medium which has higher content of nutrients and vitamins than N6 which could mediate synchronized development of shoots and roots in plantlets (Thuzar et al., 2011). All culture media were

adjusted to pH 5.75 prior to adding the gelling agent, and then autoclaved for 15 min at 121°C. For the induction of somatic embryos, the cultures were kept in the dark at 26 ± 1°C. During somatic embryo maturation and plantlet conversion, the cultures were incubated at 26 ± 1°C under cool-white fluorescent lamps (50 to 60 µmol $m^{-2}$ $s^{-1}$) for a 16 h photoperiod until they were 8 to 10 cm tall.

### Histological analysis

In order to examine the histological events associated with somatic embryogenesis, tissue samples at significant developmental stages were collected during 3 to 24 weeks of culture. The samples were fixed for 48 h in FAA solution (5 ml formalin, 5 ml glacial acetic acid, and 90 ml 50% ethyl alcohol), dehydrated through an ethanol series (30 to 90%), and embedded in paraffin wax. Serial sections of 10 µm thickness were cut using a manual microtome, and stained with Safranin O and Fast Green (Sass, 1958). The sections were mounted on slides and examined under a light microscope.

### Deoxyribonucleic acid (DNA) extraction and simple sequence repeat (SSR) analysis

The regenerated plantlets derived from 120 mg/L 2,4-D culture medium were used for SSR analysis. For DNA extraction, leaf tissues of the plants were randomly selected from six lines; with each line consisting of 7 leaf samples (one culture plant from 1st regeneration and its six regenerated plantlets). These six lines were obtained from the cultures of six zygotic embryos. DNA was extracted from young leave using a modified CTAB method (Doyle and Doyle, 1990). One hundred mg of leaf tissues were ground with a mortar and pestle in liquid nitrogen, transferred to a 1.5 ml microtube containing 700 µl of DNA extraction buffer [2% (w/v) cetyl trimethyl ammonium bromide (CTAB); 1.4 M NaCl; 100 mM Tris-HCl; 25 mM EDTA, pH 8.0; 6% (w/v) PVPP; and 40 µl ß mercaptoethanol] and incubated at 60°C for 30 min; thereafter the samples were incubated on ice for 10 min. Organic extraction was carried out by adding 300 µl of 5 M potassium acetate, mixing well, incubating on ice for 30 min, and centrifuging at 12,000 rpm for 15 min. Then, 700 µl of the upper phase was transferred to a new microtube; 700 µl of chloroform–isoamyl alcohol (24:1) was added to each sample, mixed for 20 min, and centrifuged at 12,000 rpm for 15 min. The supernatant was transferred to a new microtube with 900 µl of 100% ethanol added, and then centrifuged at 12,000 rpm for 15 min. After centrifugation, the ethanol was discarded and the DNA pellet was washed with 1.0 ml of 70% ethanol. The pellet was then dried at room temperature, diluted in 50 µl of HyClone water, quantified in 1% agarose gel, and stored at -20°C until use.

This study was conducted for six lines; the regenerated plantlets were compared with their respective culture plants in all six lines. The genetic relationship among individuals was assessed using 20 SSR markers which are distributed on all 16 linkage groups, as in the method developed by Billotte et al. (2005). Among these, five primer pairs (mEgCIR3275, mEgCIR3376, mEgCIR0782, mEgCIR3519 and mEgCIR3607 which are located on linkage group 4, 8, 16, 10 and14 respectively) were selected based on the polymorphism of PCR (polymerase chain reaction) products and their quality. The sequences of the primers used are shown in Table 1. PCR reactions were performed using a KAPA2G™ Robust HotStart PCR kit (Kapa Biosystems, Boston, MA, USA) in 10 µl reaction mixture consisting of 2 µl of KAPA2G Buffer B (containing 1.5 mM $MgCl_2$), 2 µl of KAPA Enhancer, 0.5 µl dNTP (10 mM), 0.1 µl KAPA2G Robust DNA polymerase (5 units/µl), 3.9 µl sterilized distilled water, 0.5 µl of 5 µM forward and reverse primers, and 1 µl genomic DNA (50 ng) as template. The PCR was performed in a 96-well GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). After preheating at 95°C for 3 min, the PCR reaction was carried out for 35 cycles under the following conditions: denaturing at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s, followed by one cycle at 72°C for 5 min at the end of amplification. An individual aliquot from each PCR reaction was initially assessed by electrophoresis on 1% agarose gel. Samples were then analyzed by a gel-based capillary electrophoresis method, employing a high-resolution gel cartridge on a QIAxcel® system (Qiagen, Hilden, Germany).

### Data collection and statistical analysis

The present study evaluated the effects of explant position (basal, middle and apex) of regenerated plant tissues and 2,4-D concentration (100, 120 and 140 mg/L) on primary callus induction and embryogenic callus production. The experiment was a completely randomized factorial design, with four repetitions. Each repetition consisted of six Petri dishes for each 2,4-D concentration (18 Petri dishes in each repetition). Within each Petri dish, there were six pieces of explants (two pieces each from the basal, middle and apex). Primary callus induction was evaluated after 3 months of culture, and embryogenic callus induction was evaluated after 5 months of culture. Data were subjected to ANOVA analysis; significance was determined at a 95% significance level using IRRIstat 4.4 (IRRI, 2004) software.

## RESULTS AND DISCUSSION

### Effects of 2,4-D concentration and explant position on primary callus induction and embryogenic callus induction

The effects of 2,4-D and explant position on the acquisition of embryogenic competence using different tissue segments of *in vitro* plantlets regenerated from a previous experiment were examined. In our preliminary tests, culture of young leaf tissues produced higher phenolic compounds than culture of zygotic embryos. In this experiment on using young leaf tissue, we tested different concentration of 2,4-D, the most effective auxin in our genotypes, in association with AC. It resulted that AC has positive effects: minimized oxidation and adsorbed toxic phenolic compounds and oxidative products released by the explants and therefore enhanced embryogenic callus induction and somatic embryogenesis. On the other hand, addition of AC will reduce the availability of nutrients and hormones. In order to meet the minimum auxin requirement in culture, 2,4-D was increased 50 fold in the medium added with AC (Teixeira et al., 1993). Therefore, high concentration of 2,4-D (100 to 140 mg/L in the presence of AC) employed in this experiment would be a similar effect of using 2,4-D 2 to 2.8 mg/L. The highest primary callus induction rate (27.8%) was observed on basal medium with 2,4-D 120 mg/L. It was significantly higher than the induction rate on media with 2,4-D 100 and 140 mg/L (13.9 and 11.1%, respectively). In terms of explant position, the basal segment showed the highest callus induction rate (36.1%), which was significantly higher than the responses from the middle and apex segments

**Table 1.** The code number and sequences of primers used in this study.

| Code number | Forward primers (5'- 3') | Reverse primer (5'- 3') | Allele size (bp) |
|---|---|---|---|
| mEgCIR3275 | GAAGCCTGAGACCGCATAGA | TTCGGTGATGAAGATTGAAG | 110 |
| mEgCIR3376 | CCCTCCCTGCTACCTTCT | TTATGTGAGTGCCTTTGATG | 210 |
| mEgCIR0782 | CGTTCATCCCACCACCTTTC | GCTGCGAGGCCACTGATAC | 154-214 |
| mEgCIR3519 | CCACTGCTTCAAATTTACTAG | GCGTCCAAAACATAAATCAC | 240-354 |
| mEgCIR3607 | ATTGCAGAGATGATGAGAAG | GAGATGCTGACAATGGTAGA | 240 |

**Table 2.** Effect of explant position and 2,4-D concentration on primary callus and embryogenic callus induction.

| 2,4-D Concentration | Callus induction (%) | | | | Embryogenic callus induction (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Explant segment | | | Mean[a] | Explant segment | | | Mean[a] |
| | Basal | Middle | Apex | | Basal | Middle | Apex | |
| 100 mg $L^{-1}$ | 29.2 | 8.3 | 4.2 | $13.9^{A}$ | 8.4 | 0.0 | 0.0 | $2.8^{A}$ |
| 120 mg $L^{-1}$ | 58.3 | 20.9 | 4.2 | $27.8^{B}$ | 50.0 | 16.7 | 0.0 | $22.2^{B}$ |
| 140 mg $L^{-1}$ | 20.8 | 125 | 0.0 | $11.1^{A}$ | 12.5 | 8.4 | 0.0 | $7.0^{A}$ |
| Mean | $36.1^{c}$ | $15.5^{b}$ | $2.8^{a}$ | | $23.6^{c}$ | $8.4^{b}$ | $0.0^{a}$ | |
| CV (%) | 45.3 | | | | 60.3 | | | |
| F (Rep) | $1.46^{ns}$ | | | | $2.69^{ns}$ | | | |
| F (2,4-D concentration | 15.1** | | | | 30.46** | | | |
| F (Segment) | 54.3** | | | | 41.68** | | | |
| F (2,4-D concentration × segment) | 6.12** | | | | 13.63** | | | |

Ns = not significant. ** $p < 0.01$ (highly significant). [a]Means followed by different small letters in lines and capital in columns are statistically different at $p < 0.05$. The data are the means from six samples with four repetitions.

(15.5 and 2.8%, respectively). A similar response trend was found for the induction of embryogenic callus: 2,4-D 120 mg/L had a significantly higher influence (22.2%) when compared with 2,4-D 100 mg/L and 140 mg/L (2.8 and 7.0%, respectively). It was also observed that the basal segment showed significantly higher embryogenic callus formation (23.6%) than the middle segment (8.4%). The apex segment did not induce embryogenic callus at any of the 2,4-D concentrations.

There was a strong correlation between explant position and 2,4-D concentration for both primary callus induction and embryogenic callus induction (Table 2). The best response for both parameters was observed in 2,4-D 120 mg/L with the basal segment (58.3% for primary callus induction and 50% for embryogenic callus induction). 2,4-D contributed significantly to the enhancement of callus induction and embryogenic callus induction in oil palm (Abdullah et al., 2005; Eeuwens et al., 2002). Moreover, 2,4-D has a dual effect in *in vitro* culture by acting directly as an auxin and as a stress factor (Fehér et al., 2003). Our result was in agreement with Gaj (2004), Steinmacher et al. (2007) and Scherwinski-Pereira et al. (2010), who reported that the interplay of explant position and the concentration of auxin was a crucial factor for inducing somatic embryogenesis.

## Morphological and histological aspects of somatic embryogenesis and plant regeneration

Growth of explants and tissue swelling were observed within 2 to 3 weeks (Figure 1b). After 2 to 3 months, the outer leaf sheath of explants from the basal and middle segments became swollen, while the inner leaf tissues and/or shoot meristem showed the development of yellow or yellowish-brown primary nodular calli (Figure 1c, d). Cells from primary nodular calli were highly proliferated in the basal segment, especially in the leaf primordial tissues adjacent to the apical meristem (Figure 1e). Since the basal segment is composed of the apical meristem and the youngest leaf tissue, cells in this segment are actively dividing, so they may have greater potential to acquire embryogenic competence. In this segment, young leaf tissues adjacent to the apical meristem proliferated into primary callus (yellowish brown or light brown callus induced at 2 to 3 months of culture) and subsequently into embryogenic callus (whitish and nearly globular shaped cell aggregates/clumps protruded from the primary callus after 5 to 6 months of culture); while the apical meristem itself nearly always showed only development of primary callus (with occasional development of embryogenic callus). The outer leaf sheaths

presented a low morphogenetic response which finally turned into dry, brown-colored and scaly tissue, while the inner leaf sheaths and meristematic region induced primary calli. Regarding the *in vitro* response gradient of explant position, both vertical and horizontal gradients were observed in this study. This result is consistent with the previous report of Van Le et al. (1997). Pearly white globular staged somatic embryos were developed from the surface of primary callus after 5 months of initial culture, indicating indirect somatic embryogenesis (Figure 1f). When transferred to somatic embryo maturation medium, these embryogenic calli produced high-quality somatic embryos in a repetitive and non-synchronized manner within 3 to 5 months (Figure 1g). There were two patterns of development: somatic embryo clumps (Figure 1h), which showed a higher degree of shoot proliferation, and individual somatic embryos (Figure 1i). At 3 months, an average of 36 ± 8 somatic embryos per embryogenic callus was observed. When the cotyledonary-stage somatic embryos/embryo clumps were transferred to the plant regeneration medium, 55 ± 5% of them developed into shoots after 3 to 4 months. These somatic embryos differentiated into shoots with ranges of 6 to 40 and 4 to 32 shoots on the medium with and without 2iP, respectively. Furthermore, recurrent somatic embryogenesis was observed around the base of somatic embryo clumps or shoot clumps (Figure 1j). Plantlets 6 to 8 cm height with balanced shoots and roots were obtained after 12 to 14 months of culture (Figure 1k). The plantlets generally produced only one primary root, so these *in vitro* grown roots were pruned to 1 cm length and subcultured in the same medium; as a result, more than four new roots grew out within 3 to 4 weeks (Figure 1l). These plantlets were transferred to substrate bags for 2 months of acclimatization, and thereafter to a greenhouse where 80% of the plantlets survived after 2 to 3 months (Figure 1m).

Histological analysis of a 3-week-old basal segment cultured on 2,4-D added medium revealed that compact nodular calli developed from the apical meristem and from leaf tissues adjacent to it (Figure 2a). Cell division started to occur in cells near the perivascular tissues (Figure 2b). During the development of nodular calli, the presence of a meristematic zone was observed (Figure 2c), composed of small meristematic cells (Figure 2d). Interestingly, after 3 months of culture on callus induction medium, most nodular calli from the leaf primordial tissues had proliferated into meristematic cell masses (Figure 2e). Thereafter, these meristematic cell masses gradually developed into somatic embryos. A process was also found in which new somatic embryos were initiated from primary ones (Figure 2f). This process has certain advantages compared with primary somatic embryogenesis, such as high induction frequency, and repeatability (Dai et al., 2010). The embryogenic cells displayed a high nucleoplasmic ratio, densely stained cytoplasm, and a round nucleus with a visible nucleolus (Figure 2g). Some of these actively dividing embryogenic cells gave rise to small individualized groups (two-celled or four-celled) or proembryo aggregates enclosed by thickened cell walls (Figure 2h). The continuous proliferation of proembryos resulted in the development of somatic embryos, which subsequently matured into embryos with a well-defined protodermis (Figure 2i). Most of the well-formed somatic embryos showed continuous development with complete polarization, illustrated by the presence of procambium strands (Figure 2j and k). In the palm family, many reports have documented that the first cell division is observed in cells adjacent to the vascular tissue; this has been found in *Cocos nucifera* (Fernando et al., 2003), *Euterpe edulis* (Guerra and Handro, 1998) and *Phoenix dactylifera* (Sané et al., 2006). Our histological analysis also showed that the first cell division occurred in cells close to the perivascular tissue, and that cell proliferation continuously progressed until the formation of primary calli (composed of meristematic cells and meristematic zone). Meristematic cells intensively divided during 2 to 3 months of culture, gradually resulting in the formation of embryogenic cells which have characteristics common to meristematic cells, as postulated by Fehér (2006).

## Simple sequence repeat (SSR) analysis of regenerated plantlets

A total of five polymorphic nuclear microsatellite markers in oil palm (Billotte et al., 2005), that is, mEgCIR3275, mEgCIR3376, mEgCIR0782, mEgCIR3519 and mEgCIR3607, were selected for the assessment of clonal conformity and line uniformity in regenerated plantlets. This study was conducted for six lines, with each line consisting of 7 leaf samples (one culture plant and its six regenerated plantlets). The allele sizes of the five SSR markers ranged from 110 to 354 bp. SSR profiles for each primer revealed the same genetic identity between the 1$^{st}$ regenerated plant and its six-regenerants in all six lines. However, we observed different allele patterns between lines as the cultured plants used in this experiment were obtained from different zygotic embryos. The genetic pattern of SSR markers at the mEgCIR0782 locus between the culture plant and its six regenerated plantlets is shown in Figure 3. The results from this study showed that these five SSR markers can be used to identify the genetic relationship of regenerated plantlets with their mother tissue and to check line uniformity of clonal palms. These markers may also be potential markers for verification of the genetic origin of hybrid palms, such as popular grown cv. Tenera. Moreover, to ensure clonal fidelity and quality control of *in vitro* plantlets, molecular screening using SSR markers could be beneficial for commercial oil palm propagation.

## Conclusion

This study demonstrated a successful procedure for

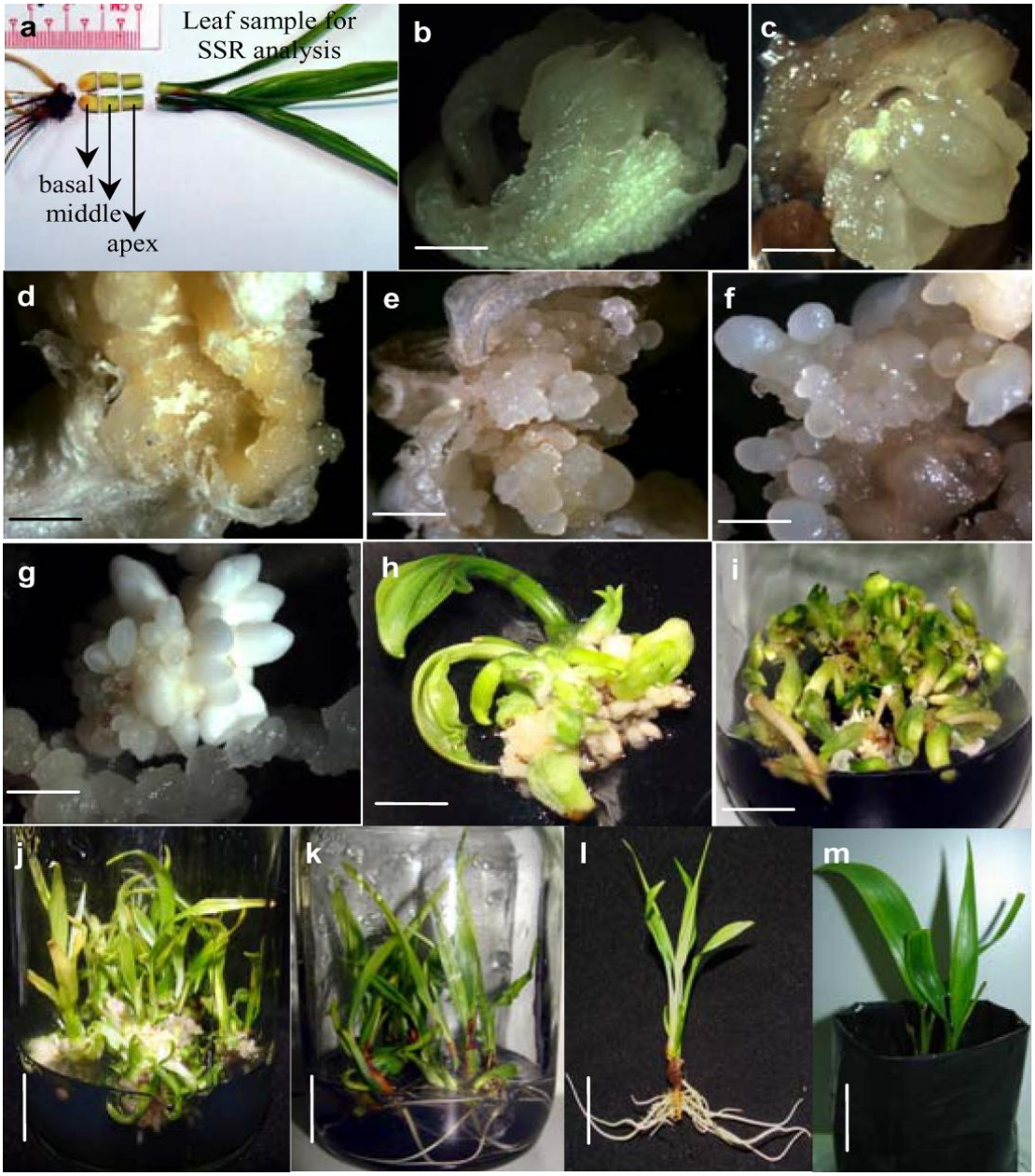


**Figure 1.** Morphological aspects of regenerated plantlet via somatic embryogenesis in oil palm: (a) schematic diagram showing explants sampling (5 mm length segments: basal, middle and apex) and SSR analysis; (b) growth of basal segment after 3 weeks culture (bar 1.7 mm); (c, d) primary callus induction in middle and basal segments after 3 months culture (bars c 2.2 mm, d 3.8 mm); (e) callus proliferation from the basal segment after 4 months culture (bar 4.5 mm); (f) progression of globular-stage somatic embryos after 5 months culture (bar 2 mm); (g) development of polyembryoids (bar 3.5 mm); (h, i) cotylendonary staged somatic embryos, in clumps and individually (bar 8 mm); (j) developing shoots in plant regeneration medium (bar 12 mm); (k) regenerated plantlets at 12 months culture (bar 16 mm); (l) *in vitro* plantlet ready for acclimatization (bar 22 mm); (m) regenerated plantlets established in the soil (bar 24 mm).

somatic embryogenesis and plant regeneration in oil palm, using the basal part of young regenerated plantlets. This culture procedure can be implemented within a relatively short time, 12 to 14 months. Our regeneration system can be applied for the cloning/recloning of elite genotypes and *in vitro* propagation of high yielding superior hybrid 'Tenera' seedling which is the most commercially grown variety in oil palm industry. In our oil palm populations, although the use of more SSR markers is still required for conclusive genetic evidence, these five

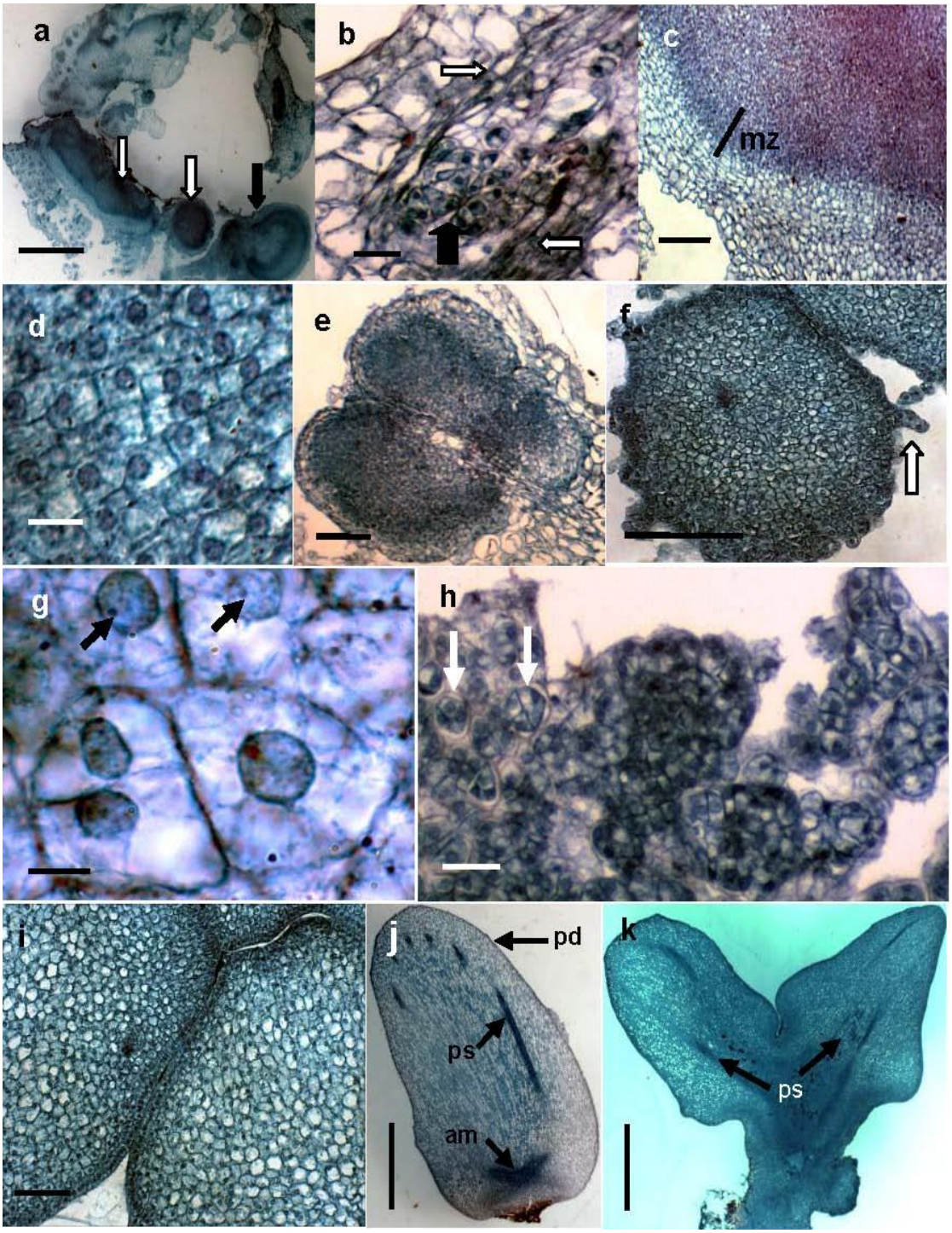


**Figure 2.** Histological analysis during oil palm somatic embryogenesis (a) basal segment tissue 3 weeks of culture (bar 1.2 mm): formation of primary calli in the apical meristem (black arrow) and along the leaf primordial tissues (white arrows); (b) initial cell division (black arrow) observed in cells adjacent to the perivascular tissue (white arrows) (bar 200 µm); (c) nodular callus forming a meristematic zone (mz) (50 µm); (d) close-up view of cells from the meristematic zone (50 µm); (e) meristematic cell mass formation (bar 200 µm); (f) embryogenic callus formation (arrow new embryos that recurred from the primary embryo); (g) magnification of embryogenic cells with high nucleoplasmic ratio, high density stained cytoplasm and round nucleus with a vissible nucleolus (arrow) (bar 10 µm); (h) embryogenic aggregates and proembryos (arrows) (bar 100 µm); (i) somatic embryo maturation with defined epidermis (bar 200 µm); (j) longitudinal section of mature somatic embryo evidencing apex meristem (am), procambial strands (ps) and protodermis (pd); (k) two somatic embryos apparently fussed with each other (j, k bars 1.5 mm)

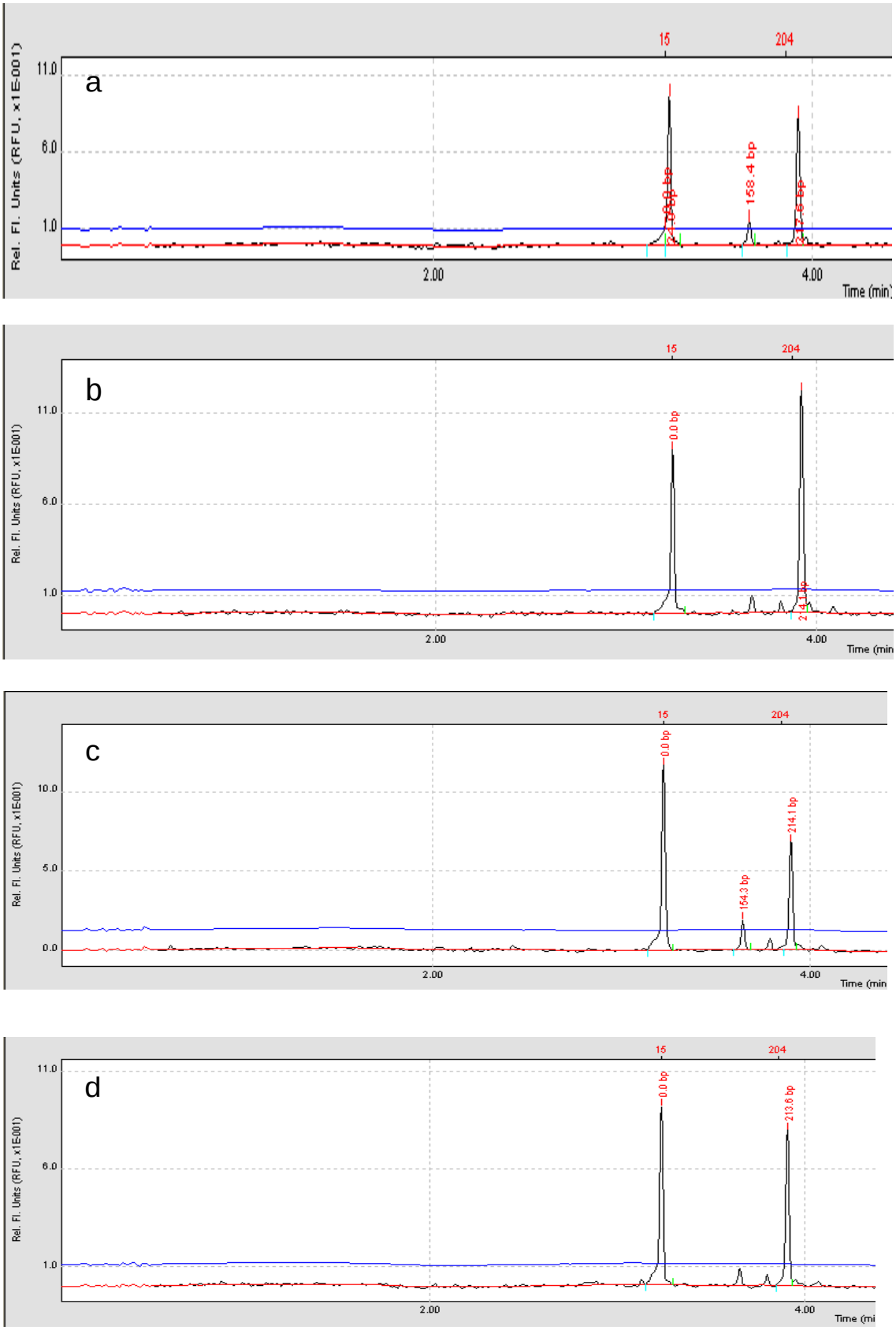


**Figure 3.** Electropherograms of SSR markers at the mEgCIR0782 locus showing the presence of the 214 bp allele in one representative line: (a) the culture plant; (b to g) its six regenerated plantlets.

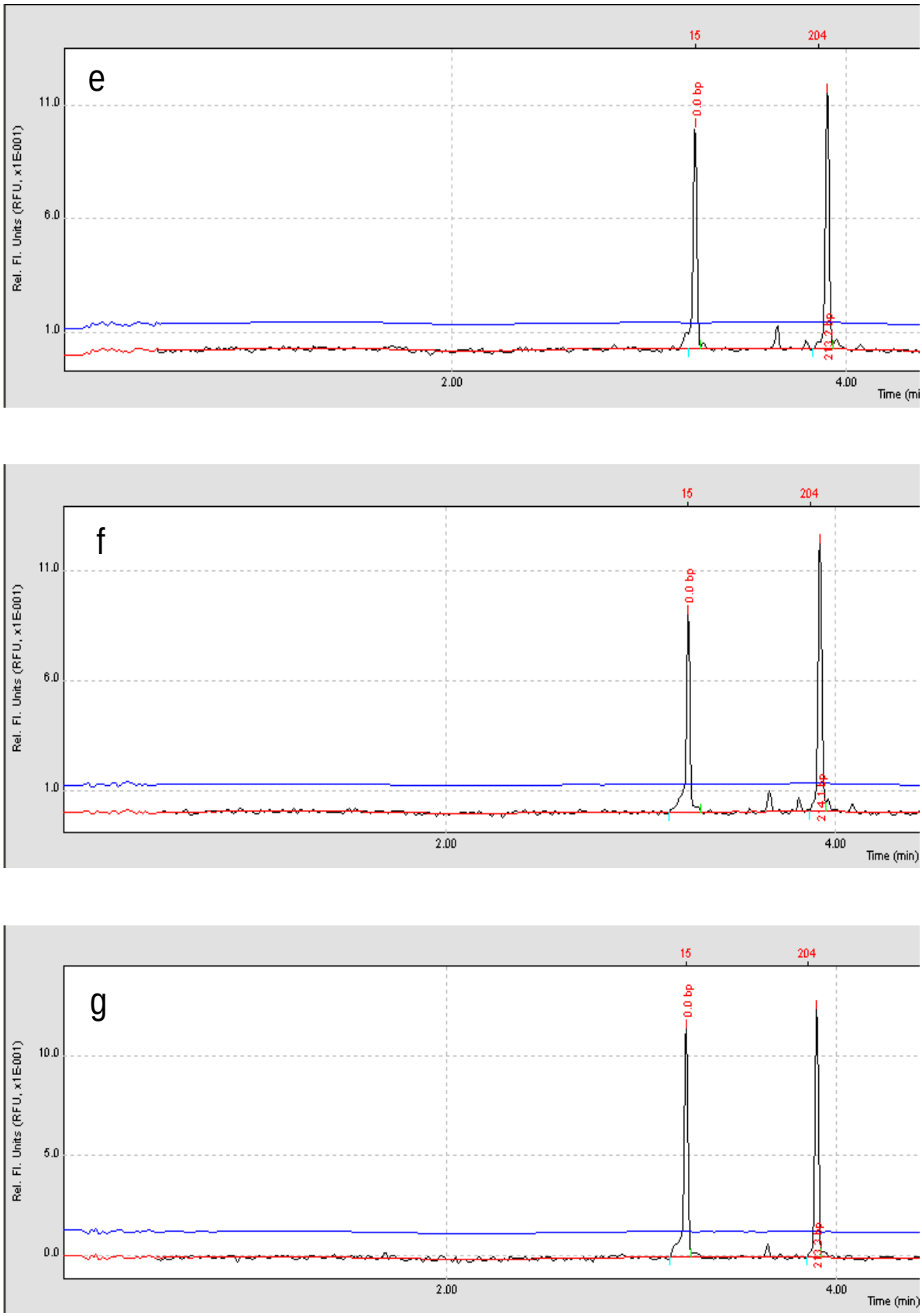


**Figure 3.** Contd.

SSR markers appear to be suitable for confirming the genetic relationship, clonal identity and uniformity of *in vitro* plants before transferring to the field. Furthermore, this work could potentially be integrated into breeding, genetic transformation and germplasm conservation programs for this important oil-bearing crop.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge: ARDA (Agricultural Research Development Agency), Thailand, for research funding; and OWSDW (Organization for Women in Science for the Developing World), Italy, through a postgraduate fellowship to Ms. Mya Thuzar.

## Abbreviations

**2iP,** 2-Isopentyladenine (6-dimethylaminopurine); **2,4-D,** 2,4-dichlorophenoxyacetic acid; **AC,** activated charcoal; **SSR,** simple sequence repeat.

### REFERENCES

Abdullah R, Zainal A, Heng WY, Li LC, Beng YC, Phing LM, Sirajuddin SA, Ping WYS, Joseph JL, Jusoh SA, Muad MR, Huey YL (2005). Immature embryo: A useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. Electron J. Biotechnol. 8:24-34.

Billotte N, Marseillac N, Risterucci AM, Adon B, Brottier P, Baurens FC, Singh R, Herran A, Asmady H, Billot C, Amblard P, Durand-Gasselin T, Courtois B, Asmono D, Cheah SC, Rohde W, Ritter E, Charrier A (2005). Microsatellite-based high density linkage map in oil palm (*Elaeis guineensis* Jacq.). Theor. Appl. Genet.110:754-765.

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sinica 18:659-668.

Dai JL, Tan X, Zhan YG, Zhang YQ, Xiao S, Gao Y, Xu DW, Wang T, Wang XC, You XL (2010). Rapid and repetitive plant regeneration of *Aralia elata* Seem. via somatic embryogenesis. Plant Cell Tissue Org. Cult. 104:125-130.

Doyle JJ, Doyle JL (1990). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15

Eeuwens CJ, Lord S, Donough CR, Rao V, Vallejo G, Nelson S (2002). Effects of tissue culture conditions during embryoid multiplication on the incidence of “mantled” flowering in clonally propagated oil palm. Plant Cell Tissue Org. Cult. 70:311-323.

Fehér A, Pasternak TP, Dudits D (2003). Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Org. Cult. 74:201-228.

Fehér A (2006). Why somatic plant cells start to form embryos? In: Mujib A, Samaj J (eds), Somatic embryogenesis. Springer, Heidelberg pp.85-101.

Fernando SC, Verdeil JL, Hocher V, Weerakoon LK, Hirimburegama K (2003). Histological analysis of plant regeneration from plumule explants of *Cocos nucifera*. Plant Cell Tissue Org. Cult. 72:281-284.

Gaj MD (2004). Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul. 43:27-47.

Guerra MP, Handro W (1998). Somatic embryogenesis and plant regeneration in different organs of *Euterpe edulis* Mart. (Palmae): control and structural features. J. Plant Res. 111:65-71.

Gueye B, Morcillo F, Collin M, Gargani D, Overvoorde P, Aberlenc-Bertossi F, Tranbarger TJ, Sane D, Tregear JW, Borgel A, Verdeil JL (2009). Acquisition of callogenic capacity in date palm leaf tissues in response to 2,4-D treatment. Plant Cell Tissue Org. Cult. 99:35-45.

IRRI (2004). IRRISTAT for Windows version 4.4: tutorial manual. Biometrics and Bioinformatics Unit, International Rice Research Institute, Manila, Philippines.

Jiménez VM, Guevara E, Herrera J, Bangerth F (2001). Endogenous hormone levels in habituated nucellar *Citrus* callus during the initial stages of regeneration. Plant Cell Rep. 20:92-100.

Rival A, Parveez GKA (2004). *Elaeis guineensis*, oil palm. In: Litz RE (ed) Biotechnology of fruit and nut crops. CABI Publishing, Wallingford, UK pp.113-143.

Sané D, Aberlenc-Bertossi F, Gassama-Dia YK, Sagna M, Trouslot MF, Duval Y, Borgel A (2006). Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera*). Ann. Bot. 98:301-308.

Sass JE (1958). Botanical microtechnique, 3rd edn. Iowa State University Press, Ames IA. pp.26-50.

Scherwinski-Pereira JE, da Guedes RS, Fermino PCP, Silva TL, Costa FHS (2010). Somatic embryogenesis and plant regeneration in oil palm using the thin cell layer technique. *In vitro* cell. Dev. Biol. Plant. 46:378-385.

Singh R, Nagappan J, Tan SG, Panandam JM, Cheah SC (2007). Development of simple sequence repeat (SSR) markers for oil palm and their application in genetic mapping and fingerprinting of tissue culture clones. Asia-Pac. J. Mol. Biol. 15:121-131.

Steinmacher DA, Krohn NG, Dantas ACM, Stefenon VM, Clement CR, Guerra MP (2007). Somatic embryogenesis in peach palm using the thin cell layer technique: Induction, morpho-histological aspects and AFLP analysis of somaclonal variation. Ann. Bot. 100:699-709.

Teixeira JB, Srndahl MR, Kirby EG (1993). Somatic embryogenesis from immature zygotic embryos of oil palm. Plant Cell Tissue Org. Cult. 34:227-233.

Thuzar M, Vanavichit A, Tragoonrung S, Jantasuriyarat C (2011). Efficient and rapid plant regeneration of oil palm zygotic embryos cv. ‘Tenera’ through somatic embryogenesis. Acta Physiol. Plant. 33:123-128.

Van Le B, My Nghieng Thao D, Gendy C, Vidal J, Tran Thanh Van K (1997). Somatic embryogenesis on thin cell layers of a $C_4$ species, *Digitaria sanguinalis* (L.) Scop. Plant Cell Tissue Org. Cult. 49:201-208.

Wang ZY, Ge Y, Mian R, Baker J (2005). Development of highly tissue culture responsive lines of *Lolium temulentum* by anther culture. Plant Sci. 168:203-211.