REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA) FOR THE IDENTIFICATION OF DNA MARKERS ASSOCIATED WITH TISSUE CULTURE AMENITY IN OIL PALM

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ABSTRACT

Tissue culture is a promising technology for mass propagation of high yielding oil palm. However, the large scale production of clonal palm is still not optimal. The application of DNA marker for early diagnosis of high quality ortets (selected oil palm tree for cloning) would greatly help in reducing the production cost. Representational difference analysis (RDA) was used to identify genomic differences between the oil palm explant tissues of the same genotypes but producing different somatic embryogenesis rates in culture. The putative RDA products isolated were cloned, sequenced and verified. Among the products isolated, C1-20 and C14-19 from forward and reverse RDA respectively, possess a single nucleotide change when aligned with the equivalent DNA region of the subtracted tissues. However, further verification is needed to confirm the discriminating effects of these products on the fecundity of the selected ortets.

Keywords: oil palm, tissue culture, representational difference analysis (RDA), DNA marker.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocotyledonous plant that belongs to the family Arecaceae (Corley and Tinker, 2003). It is the most productive of all oil crops with an average yield of about 3-4 t mesocarp (palm) oil per hectare per year in contrast to yields of most competing oil crops which are typically less than 1 t ha^{-1} yr^{-1} (Murphy, 2007; Wahid *et al.*, 2005). In Malaysia, the oil palm has been extensively cultivated over decades to meet the increasing demand for food and non-food products. With limited agricultural land however, there is an urgent need to increase productivity in terms of yield and profitability per hectare. Since the production of superior hybrid *tenera* seeds has been progressing very slowly as each selection cycle lasts for around 10 years (Jouannic *et al.*, 2005), multiplying elite palms from the best genotypes through tissue culture is seen as an alternative approach to shortcut the process (Kushairi *et al.*, 2006; Duval *et al.*, 1995). Moreover, the promise of significant oil yield increase by planting the clonal palms instead of the *dura* x *pisifera* seed palms has been proven (Sharifah and Abu, 2007; Khaw and Ng, 1997; Donough and Lee, 1995).

The current production capacity of clonal palms is still low. The formation of callus and somatic

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embryos remains the major bottleneck in oil palm tissue culture with average rates of 19% and 3% - 6% respectively. Furthermore, only 50% of the embryoids are able to proliferate actively (Corley and Tinker, 2003). One of the strategies in addressing the above problems is the incorporation of markers in the oil palm tissue culture process (Rajinder *et al*., 2009). These markers can be applied as a diagnostic tool to screen for high quality ortets which are more amenable to the tissue culture process. Thus, with a proper screening for these amenable ortets, the overall cost is expected to be reduced as compared to selecting random ortets.

Representational difference analysis (RDA), first described by Lisitsyn *et al.* (1993), is a powerful technique for identifying the differences between two nearly identical genomes (Baldocchi and Flaherty, 1997). This technique combines the use of representations with subtractive hybridisation and kinetic enrichment and results not only in the isolation of DNA fragments that are found in one of the two genomes, but also identification of differences in the restriction sites between two genomes (Lisitsyn, 1995). RDA has a broad range of applications where it has been applied to detect genetic abnormalities in cancer cells (Yuan *et al.,* 2003), species identification (Nekrutenko *et al.,* 1999) and in the identification of differences between bacterial strains (Allen *et al.,* 2003; 2001; Calia *et al.,* 1998).

The overall aim of this study was to identify unique genomic differences between ortets that exhibited different rates of somatic embryogenesis in the oil palm tissue culture process. The identified DNA region could then be developed into DNA markers for the diagnosis of ortets with good tissue culturability performance.

MATERIALS AND METHODS

Experimental Design and Plant Materials

In RDA, the sample in which unique sequences are being sought is designated as a tester and the sample whose genome is being subtracted from the tester is referred to as the driver. Reciprocal RDA subtractions were performed with young leaves of a highly prolific ortet/parent palm known as F4520 being the tester (T) and young leaves of a low prolific ortet/parent palm known as F4519, being the driver (D) in one experiment, and *vice versa* in the other experiment. Both ortets were of La Me background and the young leaves were provided by FELDA Agricultural Research Sdn Bhd (Malaysia). Ortet selection for this study was based on the previous performance of these palms in tissue culture, in terms of embryogenesis rate per ortet cultured. These ortets were resampled for tissue culture and their performance through this round of tissue culture was classified into two groups: low and high prolific. High prolific ortets produced an embryogenesis rate of more than 4%, while the embryogenesis rates for low prolific ortets ranged from 0.1% - 3.9%.

DNA Extraction

Genomic DNA was isolated from 100 mg young leaf tissue using the procedure described by Dellaporta *et al.* (1983).

Representational Difference Analysis (RDA)

RDA was carried out following the procedures described by Lisitsyn (1999) with minor modifications as detailed.

For the preparation of T and D amplicons, 1 mg of T and D DNA were digested to completion with 20 units of the *Bgl*II restriction enzyme (New England Biolabs, UK). The *Bgl*II digests were then extracted with phenol/chloroform, precipitated and dissolved in water. Following that, the digests were ligated to the adaptor pair RBgl12 (5′ GATCTGCGGTGA 3′) and RBgl24 (5′ AGCACTCTCCAGCCTCTCACCGCA 3′). The ligated products were amplified by polymerase chain reaction (PCR) using RBgl24 as a primer. PCR amplification was performed using the following programme: 94°C for 3 min; 30 cycles at (94°C for 1 min; 72°C for 3 min) and 72°C for 10 min. All amplicons were then digested with *Bgl*II to remove the RBgl24 adaptors from the ends of amplicons. Before commencing the hybridisation/amplification step, a new adaptor pair, JBgl12 (5′ GATCTGTTCATG 3′) and JBgl24 (5′ ACCGACGTCGACTATCCATGAACA 3′) was ligated only to the T DNA.

For the subtractive hybridisation, the T and D amplicons were mixed in a ratio of 1:100 (30 ng T: 3000 ng D) in a final volume of 4 μl hybridisation buffer consisting of 30 mM EPPS [(2-hydroxyethyl piperizine)-N′-(3-propane sulfonic acid)], pH 8, and 3 mM EDTA and overlaid with light mineral oil. The DNA was denatured at 95°C for 5 min, 1 μl of 5 M sodium chloride was added to a final concentration of 1 M and the reaction was incubated at 67°C for 20 hr. The hybridisation mixture was then diluted to 200 μl and an aliquot was amplified using JBgl24. The purified PCR products were digested with mung bean nuclease to remove the single-stranded DNA and re-amplified. The resulting amplicons (first round RDA products) were digested with *Bgl*II to remove the adaptor and then, ligated to another adaptor pair, NBgl12 (5′ GAT CTT CCC TCG 3′) and NBgl24 (5′ AGG CAA CTG TGC TAT CCG AGG GAA 3′) prior to the second round of hybridisation. In total, three rounds of hybridisation/amplification were performed. The second and third rounds of

hybridisation used a tester: driver ratio of 1:1000 and 1:1000 000 respectively, while the amount of the D DNA was kept constant.

Cloning of RDA Products and Sequence Analysis

The products of RDA were cloned into pCR2.1-TOPO Vector (TOPO TA Cloning Kit, Invitrogen, USA) according to the manufacturer's instructions using half of all volumes recommended. Plasmids carrying the insert were selected for plasmid isolation and sequenced. The sequences were searched against the non-redundant (nr) database using BLASTN and BLASTX programmes (http://www.ncbi.nlm.nih.gov/BLAST). A score value of more than 80 and E-value of lower than 1×10^{-5} (Verdun *et al.*, 1998) were employed as cut-off points to determine the significant similarity from the BLAST search.

Southern Blot Hybridisation

Forty micrograms genomic DNA of T and D samples were digested with *Bgl*II, electrophoresed on 0.8% (w/v) agarose gel overnight and transferred to nylon membrane (Osmonics Inc., USA). Selected products of RDA were used as probes and radioactively labelled using the High Prime labeling kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. The blot was hybridised in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% (w/v) SDS, 1% (w/v) BSA and 100 mg ml^{-1} denatured herring sperm DNA overnight at 60°C. After washing, the membrane was exposed to an imaging plate (FujiFilm) and scanned using a Phosphorimager (FujiFilm FLA5100).

Primer Design, PCR and PCR Products Analysis

Table 1 shows the primer sequences that were designed based on RDA products sequences using the program, Primer3 (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). These primers were used in a PCR reaction with DNA from palms showing high and low rate of somatic embryogenesis (from samples used in RDA) as template. PCR mixture in a total volume of 20 µl contained 100 ng of genomic DNA, 1 X PCR buffer (MPOB, Malaysia), 0.2 mM dNTP mix (Promega, USA), 0.5 µM primer and 2.5 U m*Taq* DNA polymerase (MPOB, Malaysia). The PCR amplification was carried out with the following parameters: 95°C for 3 min; 35 cycles at (95°C for 30 s; 62°C for 30 s and 72°C for 30 s) and 72°C for 7 min. The products from PCR amplification were cloned, sequenced and the fragments derived from one particular primer were aligned (T *vs*. D).

RESULTS

The products of RDA obtained after first, second and third rounds of both forward and reverse RDA experiments were analysed on agarose gel (*Figure 1*). The results revealed that the RDA products were detectable after the second round, while a clearer profile was obtained after the final round of hybridisation/amplification. RDA products from the third (final) rounds of RDA were directly cloned. A total of 44 clones (22 clones from each forward and reverse RDA) were sorted, based on size and four single clones representing the different group-sizes from both RDA were sequenced. The sequences were analysed with both BLASTN and BLASTX to determine any related sequences in the nr databases of GenBank. However, none of the sequences had significant similarity with BLASTN searches. On the other hand, through BLASTX searches, only three sequences possessed a score value and an E-value that was higher than the cut-off point; C1-20 showed similarity with the putative 1-aminocyclopropane -1-carboxylic acid oxidase of *Oryza sativa*, while C15-20 and C14-19 shared a similarity with the CCAAT-box binding factor HAP5 homolog of *Daucus carota* and integral membrane family protein of *Arabidopsis thaliana* respectively (*Table* 2).

Using these three putative RDA products as probes in the Southern hybridisation experiment, true RDA products were then verified based on the different patterns of hybridisation between their respective T and D samples (Nekrutenko *et al.*, 1999). Southern blot analysis revealed slight differences in the hybridisation pattern of the respective T and D DNA of C1-20 and C14-19. In the autoradiograph of C1-20 (*Figure 2A*), a ~3.5 kb fragment was detected in the T genome, while a fragment with a slightly higher molecular weight (~3.8 kb) was observed in the D genome. On the other hand, the autoradiograph of C14-19 (*Figure 2C*) displayed the presence of ~5.5 kb fragment in the T genome which was slightly higher than the fragment detected in the D genome (~5.0 kb). A similar hybridisation pattern between T and D genomes was observed for C15-20 (*Figure 2B*), indicating that there were no genomic differences between the two genomes. The differences in the hybridisation pattern as described, suggested that C1-20 and C14-19 fragments were true RDA products, while the C15-20 fragment was assumed as a false positive.

Further verification was carried out using PCR. Here, primers were designed based on the RDA product sequences. This PCR reaction was performed to determine whether the RDA products/primers were useful in distinguishing between their respective T and D genomes. However, for each RDA product/primer, results revealed that the PCR products were present in both genomes (*Figure 3*),

TABLE 1. THE PRIMER SEQUENCES DESIGNED BASED ON THE SEQUENCES OF THE REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA) PRODUCTS

Primer's name	Sequences 5′-3′	Expected size (bp)
C1-20 For	5′ CGA GAA CCT TGG ACT GGA AA 3′	164
C1-20 Rev	5′ GGT CAT CCT GGA GCA ATA GG 3′	
C15-20 For	5′ CAA GAG GAG GAC ACT CCA GAA 3′	158
C15-20 Rev	5′ AGT TGG CAG GAC CAC CTA TG 3′	
C14-19 For	5′ TTC CGA ATA CAT TGC ACA CC 3′	179
C14-19 Rev	5′ AGC GAC ATC GAC TGT GAA GA 3′	

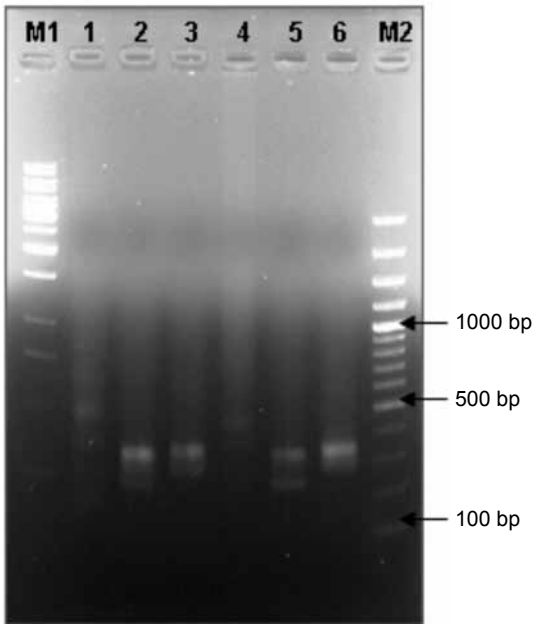

Figure 1. The representational difference analysis (RDA) products obtained from forward (lanes 1-3) and reverse (lanes 4-6) RDA experiments and electrophoresed on 1.5% (w/v) agarose gel. Lane M1: 1kb GeneRuler™ DNA ladder (Fermentas, USA); lane 1: first-round RDA products; lane 2: second-round RDA products; lane 3: final-round RDA products; lane 4: first-round RDA products; lane 5: second-round RDA products; lane 6: Final-round RDA products; lane M2: 100 bp GeneRuler™ DNA ladder (Fermentas, USA).

thus suggesting the inability of the RDA products/primers in differentiating between the respective oil palm genomes. This failure, however, led to the question of whether the different characteristics observed in the tissue culture process of the palms possessing similar genetic backgrounds used in this study were associated with single nucleotide polymorphisms. Therefore, the PCR products were cloned, sequenced and the respective sequences obtained for each RDA product/primer were aligned (T *vs*. D). However, cloning and sequencing of C15-20 RDA product was excluded as no differences were detected in both verifications through Southern and PCR. *Figures 4* and *5* show that the PCR products generated from C1-20 and C14-19 RDA products/primers possess a single base pair difference after the comparison between their respective D and T fragments were made.

DISCUSSION

In this study, RDA was performed with the aim of identifying unique genomic DNA regions that could be developed into DNA markers for the identification of oil palm ortets with higher somatic embryogenesis rates in the tissue culture process. In general, two types of products can be identified using RDA; binary (absence/presence) differences and restriction site polymorphisms (Nekrutenko *et al.*, 1999). The former refers to the difference in product/sequence that is present in one genome (T) but absent in the other (D), while the latter which is also called polymorphic amplifiable restriction fragment (PARF) (Nekrutenko and Baker, 2003; Lisitsyn *et al.*, 1993) represents differences in the position of restriction sites in the D and T genomes. In other words, PARF is the sequence that is present in both genomes but differentially flanked by the restriction sites.

The choice of restriction enzymes is very important since they establish the representations of genomes under investigation. The use of a single restriction enzyme to create representations, as reported in this study would most likely be a limiting factor as only one subset of the whole genome was represented. On the other hand, the use of another type of restriction enzyme might be complicated. However, it could provide a useful means of obtaining different genomic fragments that are not cut into RDA-compatible fragments by one particular restriction enzyme (Lammens *et al.*, 2009; Sato and Mishina, 2003; Pastorian *et al.*, 2000) . This might lead to the production of more differential-fragments from a single subtraction. Overall, the fact that the genomes can be fractionated by different restriction enzymes has made the RDA technique

TABLE 2. SEQUENCE ANALYSIS OF THE RDA PRODUCTS THAT SHOWN SIGNIFICANT SIMILARITY TO THE NON-REDUNDANT (nr) DATABASE USING BLASTX

RDA types	Clones	Length[a] (bp)	Blastx				
			Identity	Species	Accession No.	Score	E value
Forward	C1-20	291	Putative 1-aminocyclopropane-1-carboxylic acid oxidase	*Oryza sativa*	BAD61848.1	150	4e-35
	C15-20	240	CCAAT-box binding factor HAP5 homolog	*Daucus carota*	BAD15085.1	130	4e-29
Reverse	C14-19	258	Integral membrane family protein	*Arabidopsis thaliana*	NP193297.1	86.3	7e-16

Note: [a]True length of RDA difference products after removal of oligonucleotide adaptor sequences.
RDA - representational difference analysis.

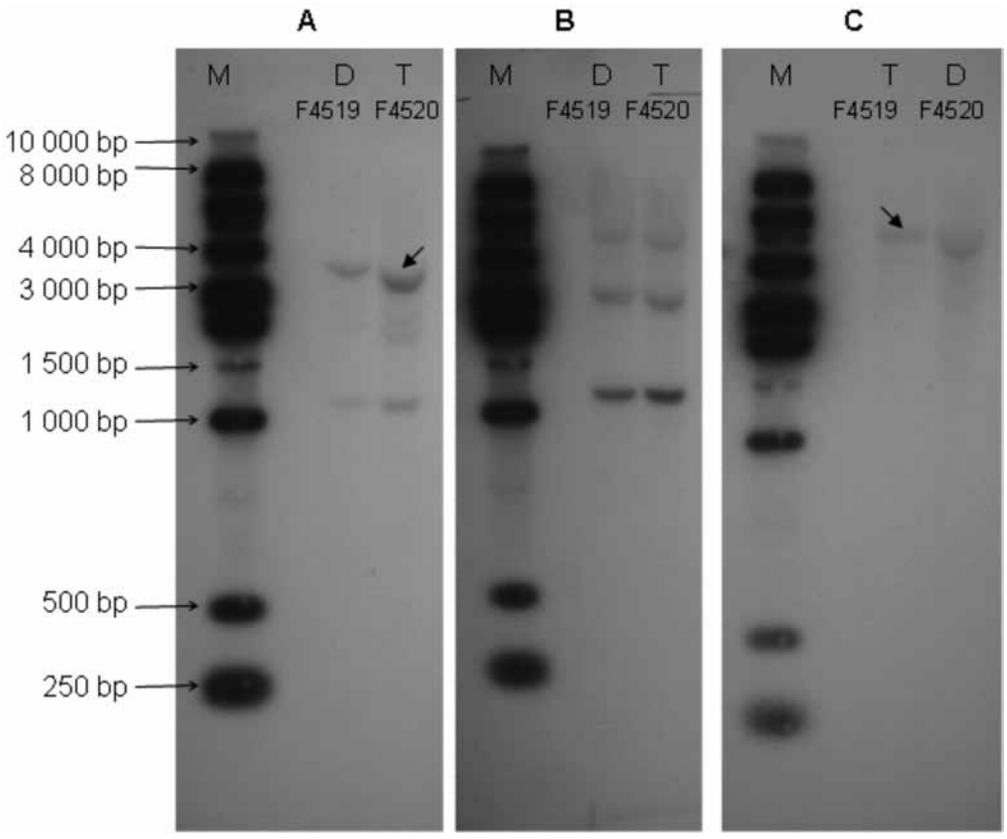

Figure 2. Southern blot analyses of the samples' genomic DNA digested with Bgl*II restriction enzyme and probed with the radiolabelled representational difference analysis (RDA) products (A: C1-20; B: C15-20; C: C14-19; T: Tester DNA; D: Driver DNA; block-arrow: fragment-size differences).*

flexible and has thus improved the chances of cloning different genomic fragments.

RDA, like many other PCR-based approaches, is prone to the isolation of false positive sequences (Pastorian *et al.*, 2000). These false positives refer to the sequences that are present in both T and D genomes or in neither of these two genomes. The former might have resulted from the inefficiency of the subtractive hybridisation step where the non-target T fragments were bound to the T fragments instead of the complement D fragments. The latter however may be caused by the contamination introduced from the repeated use of PCR (Nekrutenko *et al.*, 1999). In an attempt to eliminate such false positives, the putative RDA products must be verified using Southern blot hybridisation to ensure that the RDA products are solely hybridised to the genomes from which they were isolated.

The identification of differences due to point mutations or very small deletions or insertions in the genome might not be possible through RDA (Kunert *et al.*, 2002). However, in this study,

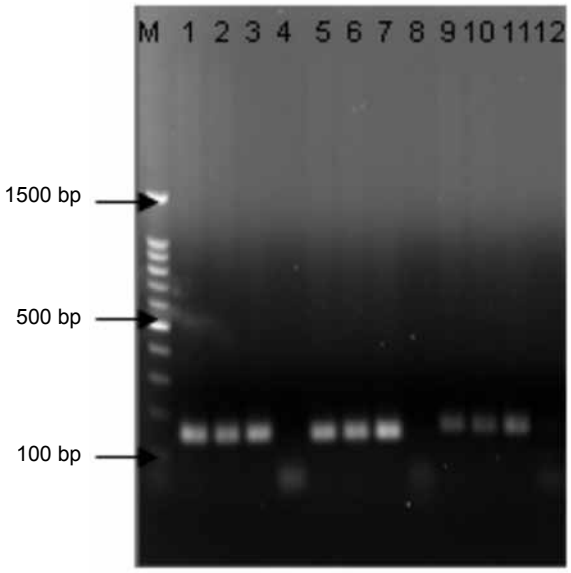

Figure 3. The polymerase chain reaction (PCR) amplified products produced from C1-20 (lanes 1-4), C15-20 (lanes 5-8) and C14-19 (lanes 9-12) primer sets on 1.5% (w/v) agarose gel. For each primer set, the lane arrangement is as followed: driver DNA, tester DNA, positive control and negative control.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
F4519 (D)       .......... .......... .......... ......CGAG AACCTTGGAC
F4520 (T)       GATCAAGTTG GCTGAAAGGC TTGCAGAACT TCTGAGCGAG AACCTTGGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
F4519 (D)       TGGAAAAGGA TTACTTGAAG AAGGCATTCT CTGAGCCATT TGTTGGAACA
F4520 (T)       TGGAAAAGGA TTACTTGAAG AAGGCATTCT CTGAGCCATT TGTTGGAGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
F4519 (D)       AAGGTGGCGA AGTACCCAAA ATGCTCTGAC CCAGAGATGG TAATGGGGCT
F4520 (T)       AAGGTGGCGA AGTACCCAAA ATGCTCTGAC CCAGAGATGG TAATGGGGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
F4519 (D)       CCGAGGCCAC ACTGATGCTG GTGGAATCAT CCTATTGCTC CAGGATGACC
F4520 (T)       CCGAGGCCAC ACTGATGCTG GTGGAATCAT CCTATTGCTC CAGGATGACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
F4519 (D)       .......... .......... .......... .......... ..........
F4520 (T)       GGGTCCCAGG TCTTGAGTTC TTAAAAGACG GGGAGTGGGT GGCAATACCT

                ....|....| ....|....| ....|....| ....|....| .
                       260        270        280        290
F4519 (D)       .......... .......... .......... .......... .
F4520 (T)       CCAGCAGAGG GTTACAGAAA TCTTTGTGAA CCTTGGTGAT C
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Figure 4. Comparison of the nucleotide sequences (tester vs. *driver alignment) of the polymerase chain reaction (PCR) amplified products using C1-20 primer set with boxed mismatched bases and shaded primer sequences.*

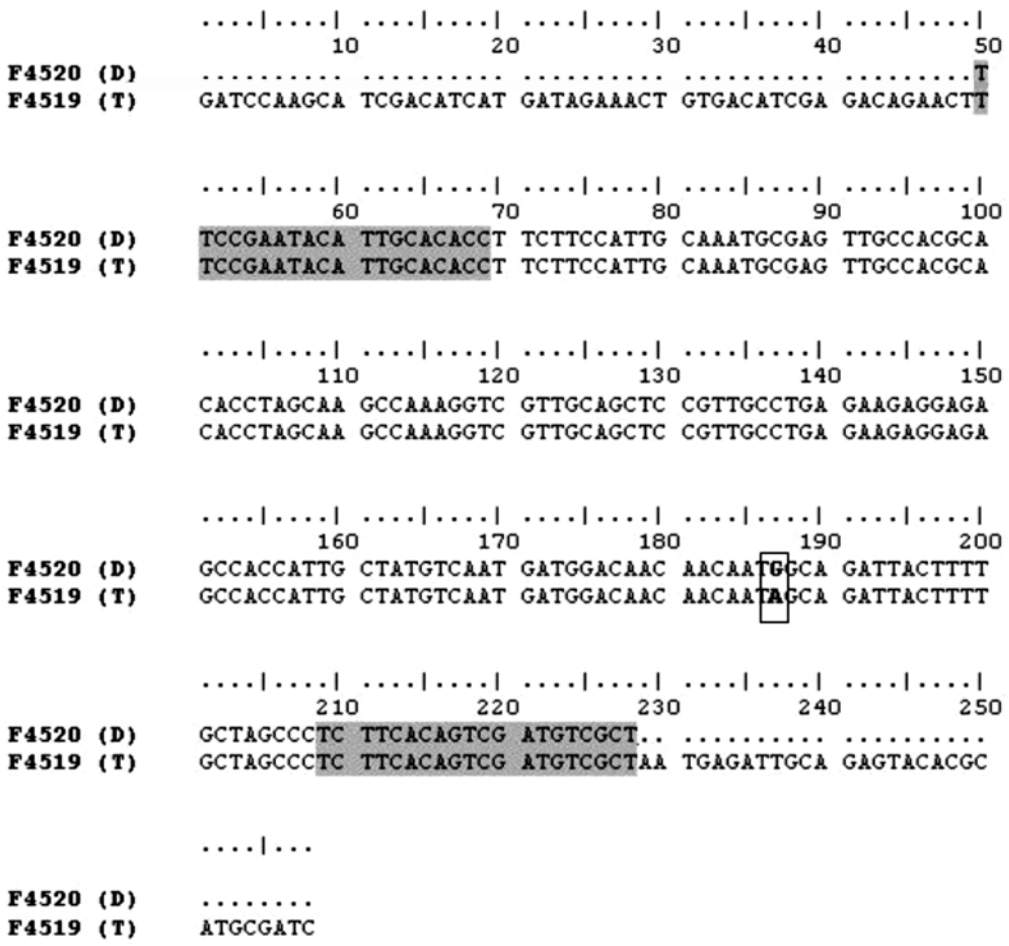

Figure 5. Comparison of the nucleotide sequences (tester vs. *driver alignment) of the polymerase chain reaction (PCR) amplified products using C14-19 primer set with boxed mismatched bases and shaded primer sequences.*

point mutations were detected from the sequence comparison (T *vs*. D) of the amplification products of primers C1-20 (high prolific-enriched fragment) and C14-19 (low prolific-enriched fragment). In the sequence comparisons made, it was noted that the base pair changes involved the changes from G→A and A→G in the T *vs*. D alignment of C1-20 and C14-19 respectively. However, nr database search using BLASTX showed that these changes represented silent mutations.

CONCLUSION

This study reports the preliminary findings of using RDA as a strategy in identifying somatic embryogenesis-related sequences. However, the current strategy can be improved by the sequencing of more RDA fragments from subtractions utilising a larger set of restriction enzymes so that the selection of informative differences can be adequately made. In addition, the sequencing of the same region in the genome of a larger set of ortets would confirm the presence of labile parts of the genome that are most likely to be modified during the tissue culture process. Furthermore, in order to ascertain whether or not the fragments identified in this study are useful as molecular markers, the expression level of the genes found should be validated with the ortets used in this experiment and in a larger set of low and high prolific ortets.

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