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GENOMIC DIFFERENCES DETECTED IN THE OIL PALM TRUNCATED LEAF SYNDROME (TLS) RAMETS (*Elaeis guineensis* Jacq.) USING THE REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA) APPROACH

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ABSTRACT

Truncated Leaf Syndrome (TLS) is a commonly found abnormality amongst tissue cultured plantlets of oil palm (Elaeis guineensis *Jacq.*) *which, if severe, will eventually lead to the death of the ramets. It was hypothesised that this phenotype could be due to genetic variability. As such, Genomic Representational Difference Analysis (G-RDA) was carried out to identify potential markers that can be used in tissue culture process for early determination of TLS ramets. A total of 18 unique sequences were successfully obtained. Primers were designed and verification of G-RDA products through polymerase chain reaction (PCR) analyses and sequence comparison was carried out using 12 clones of TLS and normal oil palm ramets. Two out of 18 set of primers [F4(6)-1181Bgl and F4(10)-1181Bgl] were identified as potential markers and further verified by PCR and Southern analyses. The primer set F4(6)-1181Bgl was only able to distinguish between TLS and normal ramet of only one genotype (Yangambi) with the presence of expected band in TLS but was absent in normal ramet. The primer set F4(10)-1181Bgl showed the presence of multiple banding pattern in the genotype of La Me and Yangambi. Analysis of the multiple band sequences revealed that those sequences represent multiple regions within the same genome, they are potentially polymorphic markers. The two primer sets mentioned above could be classified as potential genotype specific primers as it is only functional in selected genotype. Further verification with extensive number of samples is needed to elucidate the potential of the above two primer sets to be used as markers across all genotypes of oil palm.*

Keywords: somaclonal variant, tissue culture abnormality, clonal palm, DNA marker.

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INTRODUCTION

Oil palm has become the most important commodity crop in Malaysia with the increase production of palm oil from 4.1 million tonnes in 1985 to 19.2 million tonnes and its exports reached 18.1 million tonnes in 2013 (MPOB, 2014). The increasing demand for palm oil with desired characteristics is very important and this is not achievable using conventional breeding method that has a generation

period of 20 years (Abdullah *et al*., 2005). Thus, the biotechnological method such as tissue culture is the most reliable method to generate oil palm ramets (Corley and Tinker, 2003). The propagation of oil palm through tissue culture was started as early as 1960s (Corley and Tinker, 2003). In 1970s, Jones (1974) is among the first to describe the propagation of the oil palm by tissue culture method. There are several advantages shown in tissue culture of oil palm compared to the conventional breeding method, where the offspring produced via tissue culture is identical to that of original planting material and has the ability of producing new novel planting materials (Sogeke, 1998). Soh *et al*. (2001) presented that improvement in oil yield between 20%-30% is achievable using tissue culture process compared to the conventional method. However, the tissue culture process of oil palm sometimes generates somaclonal variants or abnormal palms (Corley *et al*., 1986).

The term somaclonal variation is best described as genetic variation that is observed in plantlets regenerated from tissue culture process (Matthes *et al*., 2001; Oh *et al*., 2007). Truncated Leaf Syndrome (TLS) is one of the somaclonal variations found in oil palm clonal ramets and was first reported by Tan *et al*., in 1999. The TLS is a type of vegetative abnormality which is only obvious when the tissue cultured oil palm plantlets are transferred from culture media to the nursery. In TLS ramets, stunted growth with lesser number of roots and the leaves looking like grasshopper damage are the major visible differences compared to the normal ramets, which will eventually lead to the death of the ramets, if severe. Recent phenotypic and morpho-histological analysis on TLS ramets (Habib *et al*., 2012a) reported that this plant is less vigorous compared to wild type ramets with such a characteristic of reduced height, lesser green leaves, lesser number of roots, smaller shoot apical meristem and drastically deformed stomata. Initially, Tan and colleagues (1999) suggested that the occurrence of TLS could be due to environmental factor and boron deficiency. More recently, Habib *et al*. (2012b) reported that deficiency in boron alone may not be the main cause of the TLS but also attributed by zinc deficiency too. In addition, the excessive synthesis and accumulation of cytokinins and brassinosteroids in TLS ramets, could also influence boron and zinc uptake in TLS ramets (Habib *et al*., 2012b).

However, to date there is no detailed report on the genetic differences between the TLS and wild type oil palm ramets. A number of molecular techniques are currently available to detect the genetic differences between the two genomes, such as Random Amplified Polymorphic DNA (RAPD) (Welsh and McClelland, 1990), Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al*., 1995) and Genomic Representational Difference Analysis (G-RDA) (Lisitsyn *et al*., 1993). RAPD is a PCR-based marker technique which was introduced by Welsh and McClelland (1990) and Williams *et al*. (1990) in fingerprinting of plant genomes, while AFLP is a multi-locus-based marker technique which was developed in 1995 by Vos *et al*. These two techniques are very useful in identifying random polymorphisms in plant. However, the differences between the two compared genome will be based on the concept of either presence or absence of band with the expected size rather than directly identifying the differences in the DNA sequences (Voster *et al*., 2002). Whereas, the G-RDA technique is a PCR-based genomic subtraction technique which has been widely used to clone and sequence the difference in terms of genomic losses, point mutations, genomic rearrangements or amplifications between two closely related genomes. RDA is a unique method with combination of three elements: representation, subtractive hybridisation and kinetic enrichment (Lisitsyn *et al*., 1993). Representation refers to highly reproducible DNA samples that have been digested with appropriate restriction enzyme and ligated to oligonucleotide adaptors. Subtractive hybridisation involves mixing of two closely related genomes which have been cut into small fragments and ligated to adaptors. Kinetic enrichment is an enormous enrichment and purification of target sequence. The G-RDA also has the ability to scan a complexity of about 5×10^8 base pair of DNA and was first described by Lisitsyn *et al*. (1993). Since then, the G-RDA were applied in many research involving plant genome such as DNA enrichment in a maize chromosome addition line of oat (Chen *et al*., 1998), reveal genomic differences between oak species (Zoldos *et al*., 2001), characterisation of transposable element in the genome of rice (Panaud *et al*., 2002), characterisation of sequence differences between date palm varieties (Voster *et al*., 2002), genomic changes associated with somaclonal variation (Oh *et al*., 2007) and development of markers for mantled phenotype in oil palm (Cullis and Abdullah, 2007).

The specific aim of this study was to determine the genetic differences that are present between TLS and normal oil palm ramets using G-RDA method and also to generate potential biomarkers that can be useful in the identification of TLS plantlets at the early stage of tissue culture process. Thus, improving the oil palm tissue culture efficiency, reducing cost, labour and time.

MATERIALS AND METHODS

Plant Materials and Genomic DNA Extraction

The oil palm plantlets (*Elaeis guineensis* Jacq. form *tenera*) used was derived from tissue cultured material collected from the nursery of Felda

Agricultural Services Sdn Bhd, Selangor, Malaysia. Two clones 1181 (severe and normal) and 2751 (mild and normal) were used as starting materials for genomic DNA extraction. Each clone has 20 replicates. The severity of the clones was classified based on Habib *et al*. (2012a). Genomic DNA was extracted from leaves or roots of oil palm using the method described by Dellaporta *et al.* (1983) with minor modification. The modification was at the final stage whereby DNA precipitation was performed overnight.

Genomic-Representational Difference Analysis (G-RDA)

In this study, G-RDA was carried out in two ways:

Forward RDA. The forward RDA was carried out using DNA from the abnormal ramets (TLS) as tester DNA and DNA from the normal ramets (wild type) as driver DNA. Here, only the unique sequences that were present in the tester DNA during the denaturation and re-association of DNA strands were amplified and the common sequences in both tester DNA and the driver DNA were eliminated.

Reverse RDA. The reverse RDA was carried out using DNA from the normal ramets (wild type) as tester DNA and DNA from the abnormal ramets (TLS) as driver DNA, where the unique sequences in the driver DNA were amplified and the common sequences in both driver DNA and tester DNA were eliminated.

Subtractive Hybridisation and Cloning

Genomic RDA was carried out using the method described by Lisitsyn *et al*. (1993) with minor modifications. A total of 5 μg genomic DNA (TLS and normal) were digested with 20U of restriction enzyme *BamHI* or *BglII* (New England Biolabs, UK). Four rounds of subtractive hybridisation were carried out with a tester to driver DNA ratio of 1:1000, 1:10 000, 1:100 000 and 1:1 000 000 for first, second, third and fourth rounds, respectively. The final subtractive hybridisation products were cloned into the yT&A cloning vector (Invitrogen, USA) following the manufacturer's instructions and transformed into the DH5α *E. coli* competent cells. Positive clones were selected via colony PCR and sequenced (Macrogene, China).

Sequence Analysis

The sequenced G-RDA clones (*Table 1*) were subjected to homology searches in the GenBank using the BLASTN (search a nucleotide database using a nucleotide query) and BLASTX (search a protein database using a nucleotide query) (http://www.ncbi.nlm.nih.gov/Blast).

TABLE 1. LIST OF FORWARD GENOMIC REPRESENTATIONAL DIFFERENCE ANALYSIS (G-RDA) CLONES OBTAINED FROM SEQUENCING RESULT

Ramets type	Restriction enzyme	Clone	Length [a] (bp)
1181 (Forward G-RDA)	*BamHI*	F4(1)-1181BamHI	112
		F4(2)-1181BamHI	98
		F4(3)-1181BamHI	77
		F4(6)-1181BamHI	101
		F4(10)-1181BamHI	126
		F4(14,16)-1181BamHI	98
		F4(15)-1181BamHI	90
		F4(17)-1181BamHI	104
		F4(19)-1181BamHI	88
	BglII	F4(4,14)-1181BglII	159
		F4(6)-1181BglII	214
		F4(10)-1181BglII	253
		F4(12)-1181BglII	133
		F4(12.1)-1181BglII	153
		F4(13)-1181BglII	164
2751 (Forward G-RDA)	*BamHI*	F4(7.11.21)-2751BamHI	111
		F4(10)-2751BamHI	87
	BglII	F4(23)-2751BamHI	151
		F4(9)-2751BglII	403
		F4(12)-2751BglII	434
		F4(19)-2751BglII	373

Note: [a] The true length of G-RDA difference products after removal of oligonucleotide adaptor sequences.

Verification of Genomic RDA Products

Primer design and PCR analysis. Pairs of primers were designed for the final RDA products using the primer3 Input Version 0.4.0 (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The genomic DNA from normal and TLS ramets (1181- normal and severe type; 2751- normal and mild type) were used as template for PCR amplification. A PCR mixture containing 10 ng of genomic DNA, 1× buffer for KOD hot start polymerase (Novagen, Germany), 1.5 mM $MgSO_4$ (Novagen, Germany), 0.2 mM dNTPs (Novagen, Germany) , 0.3 μM forward and 0.3 μM reverse primers (*Table 2*), 0.2 units of KOD hot start polymerase (Novagen, Germany) in a total volume of 10 μl was prepared. Amplification of PCR was performed for 2 min at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s depending on the annealing temperature of the primers, 15 s at 70°C and a final extension step of 10 min at 72°C. The PCR products were separated on a 2.0% (w/v) agarose gel, stained with ethidium bromide and visualised under UV light using a Gel Documentation System (Syngene, United Kingdom).

Verification through sequence analysis. Further analysis of the PCR products was done to identify the single nucleotide polymorphisms between the wild type and TLS oil palm ramets since the designed primers were not selective to the tester genome. The electrophoresed PCR products were excised and gel purified using a gel purification kit following the manufacturer's instruction (GeneAll, Korea). The gel purified PCR products were cloned following the same procedure described above and sequenced (Macrogene, China). The sequences were aligned (normal *vs*. TLS ramets, sequences) and compared using the BioEdit Sequence Alignment Editor Version 7.0.5.3 software.

Verification through PCR and southern analyses. A PCR reaction was carried out using the primer pairs which gave positive result (selective to tester genome) using genomic DNA of wild type and TLS oil palm ramets as template. The PCR reaction was carried out in a total volume of 20 μl, consisting of 10 ng of genomic DNA, 1× buffer for KOD hot start polymerase (Novagen, Germany), 1.5 mM $MgSO_4$ (Novagen, Germany), 0.2 mM dNTPs (Novagen, Germany), 0.3 μM forward and 0.3 μM reverse primers (*Table 2*) and 0.2 U of KOD hot start polymerase (Novagen, Germany). The cycling parameters were: 2 min at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s depending on the annealing temperature of the primers (*Table 2*), 15 s at 70°C and a final extension step of 10 min at 72°C. The PCR products were separated on 2.0% (w/v) agarose gel and transferred on positively charged nylon membrane (Amersham Biosciences, United Kingdom). The labelling of probes and detection was performed as described by the manufacturer (Thermo Scientific, EU).

RESULTS AND DISCUSSION

Genomic Representational Difference Analysis (G-RDA)

Four rounds of subtractive hybridisation of forward and reverse G-RDA were performed using the genomic DNA from normal and TLS (1181 and 2751) ramets. Theoretically, any resulting bands should be unique to the tester genome only. After the fourth round of G-RDA, the isolated enriched unique sequences for forward and reverse G-RDA of 1181 (severe and normal) and 2751 (mild and normal) ramets were found to be in the range of approximately 180-500 bp (*Figures 1a* to *1h*). The final round of forward difference products were extracted from gel, cloned separately and randomly selected colonies were sequenced. The insert sizes matched the sizes of DNA profiles (180-500 bp) observed during subtractive hybridisation. The reverse G-RDA results were not subjected to any verification process. It is simply because of our aim and the early objective of this study is to identify the unique sequences that were present in TLS ramet which can be used as a biomarker in identifying the TLS ramets in the early stage of oil palm tissue culture process.

After eliminating those poor sequences, a total of 21 sequences (*Table 1*) were subjected to homology search with those available oil palm genomic sequences in the GeneBank database using the BLASTN programme. Only one of those sequences [F4(4.14)-1181BglII] had identity to a known gene, ycf2 from oil palm genome of *Elaeis guineensis* Jacq. The rest of the sequences gave poor hits to sequences in the GenBank database due to the small G-RDA product size (~ < 500 bp). Moreover, a complete oil palm genome sequence was also not available in the public database at the time this work was performed. Hence, all the resulting sequences were sent to the Malaysian Palm Oil Board (MPOB, Malaysia) for further analysis to obtain longer fragments. Those sequences that were very short in length (< 200 bp) and do not match to any of the MPOB oil palm genomic sequences were not further investigated. As shown in *Table 3*, 18 out of 21 sequences were finally selected for verification.

Primer Design and PCR Analysis

Eighteen sets of primers (*Table 2*) were designed and used to amplify the expected size of PCR products from genomic DNA of normal and TLS ramet. Two primers, F4(6)-1181BglII and F4(10)-1181BglII revealed

TABLE 2. OLIGONUCLEOTIDE PRIMER SEQUENCES USED IN VERIFICATION VIA POLYMERASE CHAIN REACTION (PCR) ANALYSIS

Primer's name	Tm (°C)	Sequences 5′-3′	Optimised annealing temperature (°C)	Expected size (bp)
F4(1)-1181BamHI (F)	61.1	AGG TCA AGG GCG AAT TCG AGG	59.3	502
F4(1)-1181BamHI (R)	58.7	TTG TCG CAG GAC CTT GTA GGC		
F4(1)-1181BamHI (NF)	61.5	CGC GAG ATC CGA CCA TGG TTA	60.0	447
F4(1)-1181BamHI (NR)	61.5	CCC CAA GTG ATT CCC GCA AAT		
F4(2)-1181BamHI (F)	60.4	TCG GTT CTT TGA GAA AGA GGG CTT	56.0	490
F4(2)-1181BamHI (R)	54.7	CAA AGT CTC TCT CAT GGC CTC TAT		
F4(3)-1181BamHI (F)	57.9	ATA GCT AGC CAC CTC TCT CCA GCT	68.5	439
F4(3)-1181BamHI (R)	57.9	CGT ATC TGG GGT CCC CTT TTA TAG		
F4(10)-1181BamHI(F)	59.0	GCG GTG CTG GAA CTA ACC TAT TTC	65.0	454
F4(10)-1181BamHI(R)	60.8	AGA GAG AGA TGG GCA CAA CCG AA		
F4(14,16)-1181BamHI (F)	61.2	TTT CTT ATC GTG GGT GGC TTC TCC	70.0	431
F4(14,16)-1181BamHI (R)	64.0	AGC TGG CCG ATC TTC TCC TTC ACA		
F4(15)-1181BamHI (F)	55.6	GCC ACA ACT CTG CTC CAC TTT	70.0	460
F4(15)-1181BamHI (R)	67.9	TGT TGG TGC AAA AAT CCA TCG GCG		
F4(15)-1181BamHI (NF)	53.8	CTG CTC CAC TTT CTG TAA CGG	55.0	311
F4(15)-1181BamHI (NR)	55.0	CAA CAA GAA TGG AGT GCT CGA		
F4(17)-1181BamHI (F)	69.8	CGA GCT ATC CAA CGC CAT CCA AGT CCA	69.4	539
F4(17)-1181BamHI (R)	64.2	GTC AAG TCC TCG GTG CAC GGG ATG CGA		
F4(17)-1181BamHI (NF)	60.1	AAA TAT CCG TGG GTG GAG CAT GAC CCG	65.0	449
F4(17)-1181BamHI (NR)	64.9	ACG TCC GAA TCC CAC GAA GGC GGG ATG		
F4(19)-1181BamHI (F)	65.1	CGA TTC GTC ACC TCG ATA TTG GCG	70.8	440
F4(19)-1181BamHI (R)	62.8	CAG CGA TTG ACA TTC CCT CGG A		
F4(4,14)-1181BglII (F)	60.3	TGG CAA TTC CGT CAA GAT CTC TTC	65.0	446
F4(4,14)-1181BglII (R)	55.8	CTG TTC TCA TGT TTT GTG AAT AGC C		
F4(6)-1181BglII (F)	53.8	AGA ATA GGA GCC TTA TCA CTC TTG A	53.7	502
F4(6)-1181BglII (R)	54.7	CCT ATA TTC TGG ATA TCC CTG CAC		
F4(10)-1181BglII (F)	65.3	TTA TCT CCG ACT CCG GTG AGG TCT CTG	60.0	579
F4(10)-1181BglII (R)	66.5	CTC TTT CCC CAC CTT TGG CTC TGA CTG		
F4(12)-1181BglII (F)	54.0	GCA CCG TAC CCT TTA ACA ACA	54.3	500
F4(12)-1181BglII (R)	56.1	CAT GCG ACA TAC GAC ACC AAG		
F4(12.1)-1181BglII (F)	54.0	ATG AAG ATA ACC CTA TTG CTA GCC	60.0	411
F4(12.1)-1181BglII (R)	52.0	TAG AAG ATA GAA GAG CCC AGA TTC		
F4(13)-1181BglII (F)	62.4	ATG GCA AAA GAG TTG GGT GTT CGA	63.5	499
F4(13)-1181BglII (R)	57.0	CCC ATA GGG CGT ATT TAG TTT CTG		
F4(7.11.21)-2751BamHI (F)	65.4	TCA TTA TGG GAG CGA TCA CAC CGG	59.8	617
F4(7.11.21)-2751BamHI(R)	62.8	CCA TGG CAG AAG AAA GAG ATC CCC		
F4(10)-2751BamHI (F)	67.2	TTC GGA TGG CAT GGG GAG GTT TG	66.0	598
F4(10)-2751BamHI(R)	62.3	CCG AAC TCC TTT CGA ACT TCG TCA		
F4(10)-2751BamHI (NF)	61.5	GAC GTC AGA GGT GAT CGA TCG TTG	62.9	544
F4(10)-2751BamHI(NR)	62.1	TTC GAG CTT CTT CGA CAA CGA GGT		
F4(12)-2751BglII (F)	57.8	GAT GTC GCA CAT CCA GGA TCA	60.0	678
F4(12)-2751BglII (R)	52.1	GAC CTA AGG ATG TCA CAC ATC C		
F4(19)-2751BglII (F)	67.0	GAA GGA TGT CGC ACA TCC AGG ATC ATG	67.5	682
F4(19)-2751BglII (R)	62.8	GAC CTA AGG ATG TCA CAC ATC CGA GAT C		

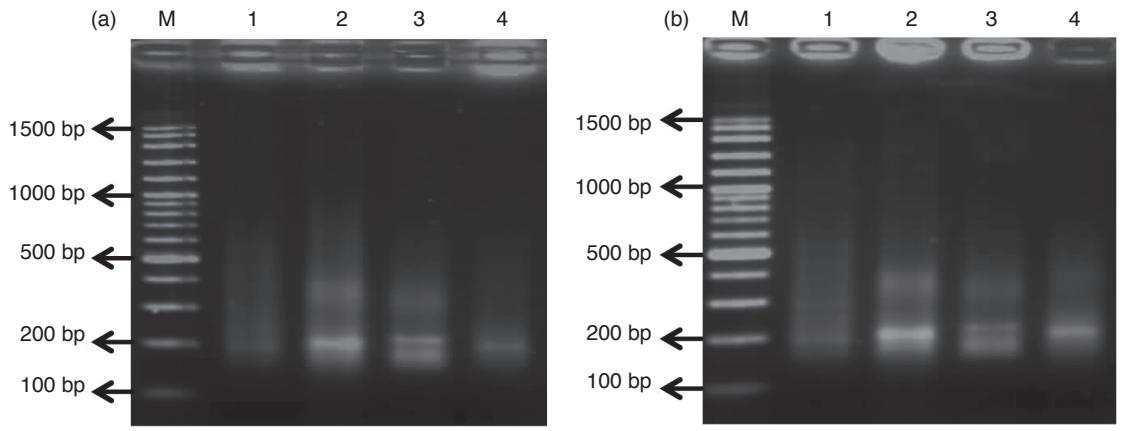

Figure 1(a and b). The difference products obtained from forward and reverse Genomic Representational Difference Analysis (G-RDA) experiment of 1181 [normal and Truncated Leaf Syndrome (TLS)] using BamHI restriction enzyme (a) and electrophoresed on 2.0% (w/v) agarose gel respectively. All the difference products were ligated to oligonucleotide adaptor sequences. Lane M contains 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lanes 1 to 4 contain first, second, third and fourth rounds of forward G-RDA respectively; lanes 5 to 8 contain first, second, third and fourth rounds of reverse G-RDA respectively.

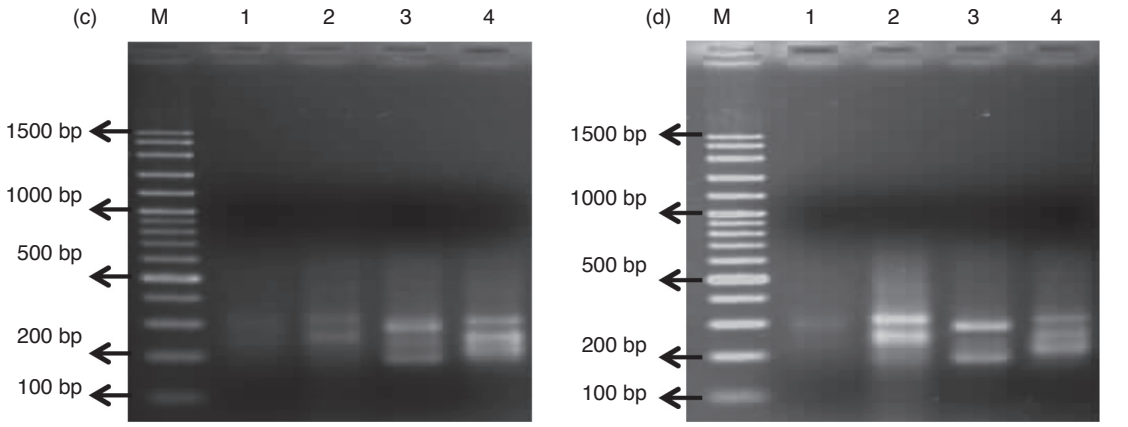

Figure 1(c and d). The difference products obtained from forward and reverse Genomic Representational Difference Analysis (G-RDA) experiment of 1181 [normal and Truncated Leaf Syndrome (TLS)] using BglII restriction enzyme and electrophoresed on 2.0% (w/v) agarose gel respectively. All the difference products were ligated to oligonucleotide adaptor sequences. Lane M contains 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lanes 1 to 4 contain first, second, third and fourth rounds of forward G-RDA respectively; lanes 5 to 8 contain first, second, third and fourth rounds of reverse G-RDA respectively.

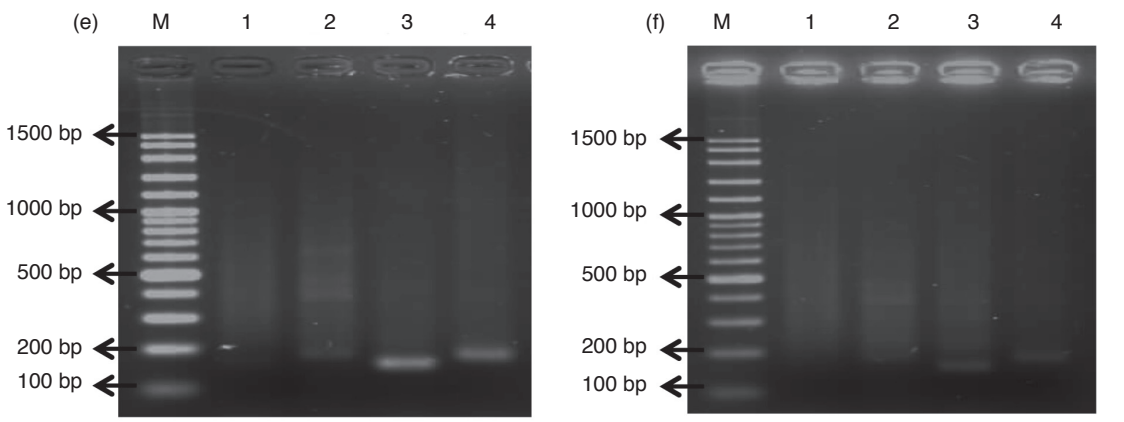

Figure 1(e and f). The difference products obtained from forward and reverse Genomic Representational Differences Analysis (G-RDA) experiment of 2751 [normal and Truncated Leaf Syndrome (TLS)] using BamHI restriction enzyme and electrophoresed on 2.0% (w/v) agarose gel respectively. All the difference products were ligated to oligonucleotide adaptor sequences. Lane M contains 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lanes 1 to 4 contain first, second, third and fourth rounds of forward G-RDA respectively; lanes 5 to 8 contain first, second, third and fourth rounds of reverse G-RDA respectively.

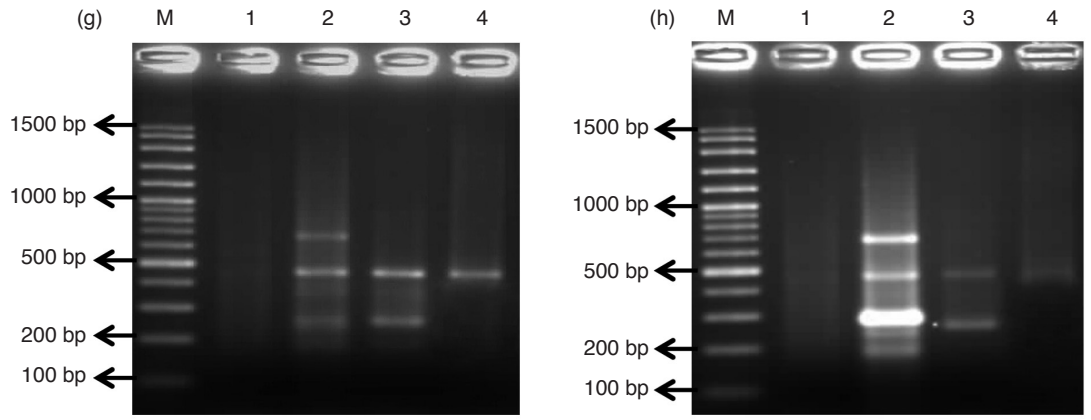

Figure 1(g and h). The difference products obtained from forward and reverse Genomic Representational Difference Analysis (G-RDA) experiment of 2751 [normal and Truncated Leaf Syndrome (TLS)] using BglII restriction enzyme and electrophoresed on 2.0% (w/v) agarose gel respectively. All the difference products were ligated to oligonucleotide adaptor sequences. Lane M contains 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lanes 1 to 4 contain first, second, third and fourth rounds of forward G-RDA respectively; lanes 5 to 8 contain first, second, third and fourth rounds of reverse G-RDA respectively.

TABLE 3. LIST OF FORWARD GENOMIC REPRENSENTATIONAL DIFFERENCE ANALYSIS (G-RDA) CLONES HITS THAT TO MPOB OIL PALM SEQUENCES

G-RDA clones	GenBank accession No.	Score	E value	Homology (%)
F4(1)-1181BamHI	ASJS01200444.1	169	$1e^{-40}$	94
F4(2)-1181BamHI	KE607421.1	145	$1e^{-33}$	93
F4(3)-1181BamHI	ASJS01158211.1	377	$1e^{-103}$	100
F4(10)-1181BamHI	ASJS01051438.1	192	$1e^{-47}$	94
F4(14,16)-1181BamHI	KE607396.1	648	0.0	89
F4(15)-1181BamHI	KE607272.1	73	$6e^{-12}$	85
F4(17)-1181BamHI	KE614597.1	176	$6e^{-43}$	97
F4(19)-1181BamHI	ASJS01169318.1	741	0.0	94
F4(4,14)-1181BglII	ASJS01038344.1	228	$3e^{-58}$	92
F4(6)-1181BglII	KE608430.1	377	$4e^{-103}$	99
F4(10)-1181BglII	ASJS01169767.1	409	$2e^{-112}$	96
F4(12)-1181BglII	ASJS01020575.1	193	$8e^{-48}$	94
F4(12.1)-1181BglII	ASJS01046557.1	250	$5e^{-65}$	97
F4(13)-1181BglII	KE608297.1	259	$1e^{-67}$	95
F4(7.11.21)-2751BamHI	ASJS01193036.1	178	$2e^{-43}$	95
F4(10)-2751BamHI	KE615082.1	134	$2e^{-30}$	94
F4(12)-2751BglII	KE607012.1	725	0.0	99
F4(19)-2751BglII	KE607012.1	689	0.0	100

significant differences between the two compared genomes (*Figure 2*). The primer set, F4(6)-1181BglII amplified the predicted 502 bp PCR product in TLS ramet but failed to amplify a fragment in normal ramet (indicated by circle in *Figure 2a*). On the other hand, primer set F4(10)-1181BglII amplified the predicted 579 bp PCR product with an additional band of 480 bp in normal ramet but failed to amplify the expected fragment in TLS ramet. However, this primer set amplified a 480 bp PCR product in TLS ramet (indicated by circle in *Figure 2b*). The rest of the primer sets did not reveal any obvious size differences in the amplified product from the two compared genomes. Lack of differences could be due to minor base pair changes or single nucleotide deletion and or insertion that might have occurred in the two compared genomes. Thus, all the resulting PCR amplified products were sequenced for further verification.

Sequence Verification

The PCR products were cloned and sequenced to identify any differences in single nucleotide or base changes possibly caused by insertions, deletions or other genomic DNA rearrangements. The complete pair wise sequence comparisons were made between the PCR product sequences of

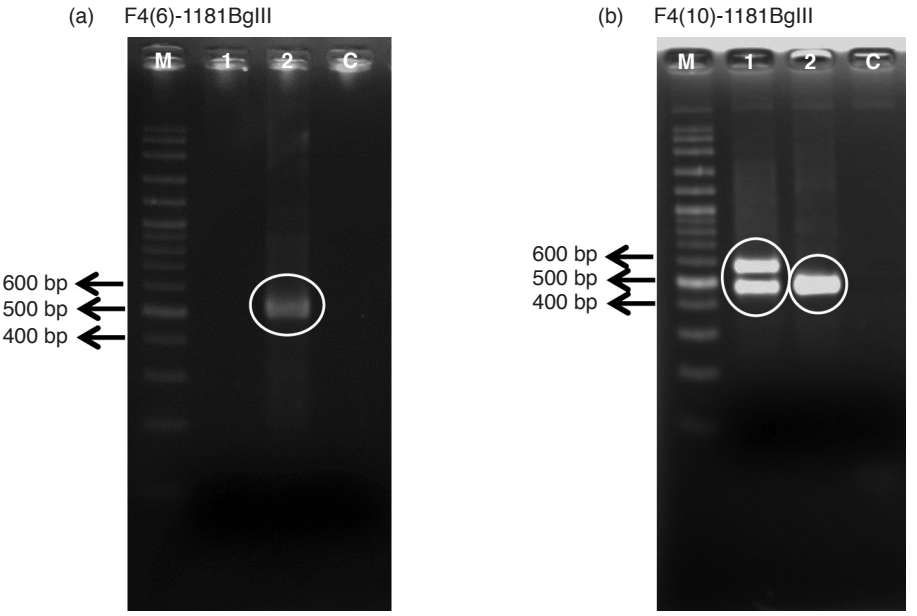

Figure 2. The polymerase chain reaction (PCR) products obtained with various sets of primers (Table 2) *designed from the selected Genomic Representational Difference Analysis (G-RDA) difference products and electrophoresed on 2.0% (w/v) agarose gel. Lane M: 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lane 1: genomic DNA from normal; lane 2: genomic DNA from TLS; lane C: negative control (without template).*

normal and TLS and the differences were scored as reported in *Table 4*.

Two primer sets, F4(12)-2751BglII and F4(19)-2751BglII showed no differences between the normal and TLS ramets (*Table 4*). This indicates that the region of difference product that was used to design primers is common to both the compared genome even though this difference product was supposed to be unique only to the TLS ramet. A possible explanation has been presented for these phenomena, where the fragments in the off-type or abnormal plants can be present in the normal plants but not all the fragments that are present in normal plants would be in the off-type or abnormal plants (Cullis and Kunert, 2000; Oh *et al*., 2007).

The sequence analysis of eight primer sets [F4(3)-1181BamHI, F4(10)-1181BamHI, F4(14,16)-1181BamHI, F4(17)-1181BamHI, F4(19)-1181BamHI, F4(13)-1181BglII and F4(7,11,21)-2751BamHI] revealed minor nucleotide or base changes in normal ramet when compared to their respective TLS ramet (< 10%, *Table 4*). A potential biomarker need to have very high sensitivity and specificity, which is at least 90% or more (Ziogas, 2011; Brower, 2011). Other than that, the eight primer sets were deemed as not potential biomarkers as they were only able to identify less than 10% nucleotide differences between the normal and TLS ramet. Phillips *et al*. (1994) suggested that nucleotide or base changes in sequences are the most common

TABLE 4. THE SUMMARY OF NUCLEOTIDE DIFFERENCES BETWEEN TRUNCATED LEAF SYNDROME (TLS) AND NORMAL GENOME OF RESPECTIVE PRIMERS SETS

Primers sets	Length (bp)	Nucleotide differences [a] (bp)	Nucleotide differences [a] (%)
F4(3)-1181BamHI	439	40.0	9.11
F4(10)-1181BamHI	454	30.0	6.61
F4(14,16)-1181BamHI	431	17.0	3.94
F4(17)-1181BamHI	449	8.0	1.78
F4(19)-1181BamHI	440	13.0	2.95
F4(6)-1181BglII	502	502	100.00
F4(10)-1181BglII	576	103	17.88
F4(10)-1181BglII	473	0.0	0.00
F4(13)-1181BglII	499	10.0	2.00
F4(7.11.21)-2751BamHI	617	2.0	0.32
F4(10)-2751BamHI	544	15.0	2.76
F4(12)-2751BglII	679	0.0	0.00
F4(19)-2751BglII	682	0.0	0.00

Note: [a] Nucleotide differences between TLS and normal genome.

point mutation observed in tissue cultured plants. Theoretically, single nucleotide or base changes will be resulting in a C to T or G to A (deamination of methylated cytosine) transition which can lead to the elimination of functional gene products of those sequences (Phillips *et al*., 1994). In this case, the most commonly observed single nucleotide or base changes is from T to C or A to G. As such, those sequences derived from these eight primer sets might be unlikely to repr esent the major cause of the abnormality that present in the TLS ramet.

Primer set F4(6)-1181Bgl showed major differences in between the normal and TLS ramet (100%, *Table 4*). This primer set was able to amplify the expected size of 502 bp only from the genomic DNA of TLS ramet which is identical to that of the final subtracted hybridisation product and unable to produce any bands in the normal ramet. This could be due to the presence of large genetic differences between the two compared genomes which prevented the primer set F4(6)-1181BglII to bind and amplify the targeted region in the genome of normal ramet.

The primer set F4(10)-1181BglII revealed differences due to deletion of approximately 100 base pair mainly in the 5' region of the TLS genome (*Figure 3*). All the three sequences retain the same forward and reverse primer as well as the same final round of G-RDA difference product sequences. This simply means that the region that was used to design the primer set F4(10)-1181BglII, is common to more than one repetitive sequences in normal ramet compared to the TLS ramet. It is unclear whether the absence of the extra region in the TLS genome might be a cause to this abnormality.

The rest of the tested primer sets showed a false positive result. The choices of tester to driver DNA ratio play an important role in the subtractive hybridisation step, where poorly chosen ratio could result in the subtraction of false positive difference product. A relatively high difference in tester to driver DNA ratio could possibly reduce the efficiency as far as 100% of targeted DNA enrichment (Cho and Park, 1998; Milner *et al*., 1995).

The resulting sequence analysis has indicated that the variations between the two tested oil palm genomes were due to single nucleotide substitution or an insertion or a deletion of a portion of sequences in either in the normal or TLS genome.

Verification through PCR and Southern Analyses

The PCR products of two primer sets [F4(6)-1181BglII and F4(10)-1181BglII] that showed significant differences between the two compared genomes (TLS and normal type) were identified as potential markers and have been further verified through PCR and Southern analysis with respect to their presence in 12 clones from different categories of TLS ramets (*Table 5*) and the resulting gel photographs of PCR product are shown in *Figure 4* [*a*(I), *b*(I), *c*(I), *d*(I), *e*(I) and *f*(I)]. The Southern hybridisation technique confirmed the presence of selected region (in this case the sequences of the final round of G-RDA product) along with a positive control (Panaud *et al*., 2002). The subtractive hybridisation product of F4(6)-1181BglII and F4(10)-1181BglII was used as a probe in Southern blot analysis against the PCR products and the resulting X-ray films are shown in *Figure 4* [*a*(II), *b*(II), *c*(II), *d*(II), *e*(II) and *f*(II)] respectively.

As shown in *Figure 4*, the normal and TLS ramet from clone 1181 is the only pair that is distinguishable using the primer set F4(6)-1181BglII [lane 1 indicated by circle in *Figure 4a* (I) and (II)]. This result suggests that the region corresponding to the probe used for hybridisation is absent exclusively in the normal ramet of clone 1181. This could be due to the differences in the plant genotype for each respective clone (Panaud *et al*., 2002; Zoldos *et al*., 2001). As shown in *Table 5*, only the paternal palm of clone 1181 was derived from the variety of Yangambi unlike the rest of the clones. Oil palm ramets that are derived from different varieties will possess different genomic sequences. Therefore, in future, the starting material and the samples for verification should include a range of variety to elucidate whether or not the designed primer can be used as a candidate biomarker to detect the TLS occurrence across various genotypes.

The primer set F4(10)-1181BglII showed a complex hybridisation pattern among the 12 clones tested as shown in *Figures 4d*, *4e* and *4f*. The first hybridisation pattern showed the presence of double bands in normal and single band in TLS genome while the second pattern showed the presence of single band in normal and double bands in TLS genome. The third and the fourth one have double bands and single band in both the normal and TLS genomes, respectively.

The primer set F4(10)-1181BglII showed similar hybridisation pattern in clone 2751 [Lanes 19 and 20 indicated by circle in *Figure 4f* (I and II)] and clone 1181 [lanes 1 and 2 indicated by circle in *Figure 4d* (I and II)] with the presence of double bands in normal and single band in TLS ramet. Even though paternal palms for both the clones (1181 and 2751) are from different genotypes which are Yangambi and La Me respectively, they still show an identical banding pattern to each other. This occurrence can be understood better if a large number of clones from Yangambi and La Me varieties are tested during the verification process. If the results are reproducible in both the Yangambi and La Me variety, these primer set can be possibly used as a potential marker for these genotypes to identify the presence of TLS ramets at the early stage of oil palm tissue culture.

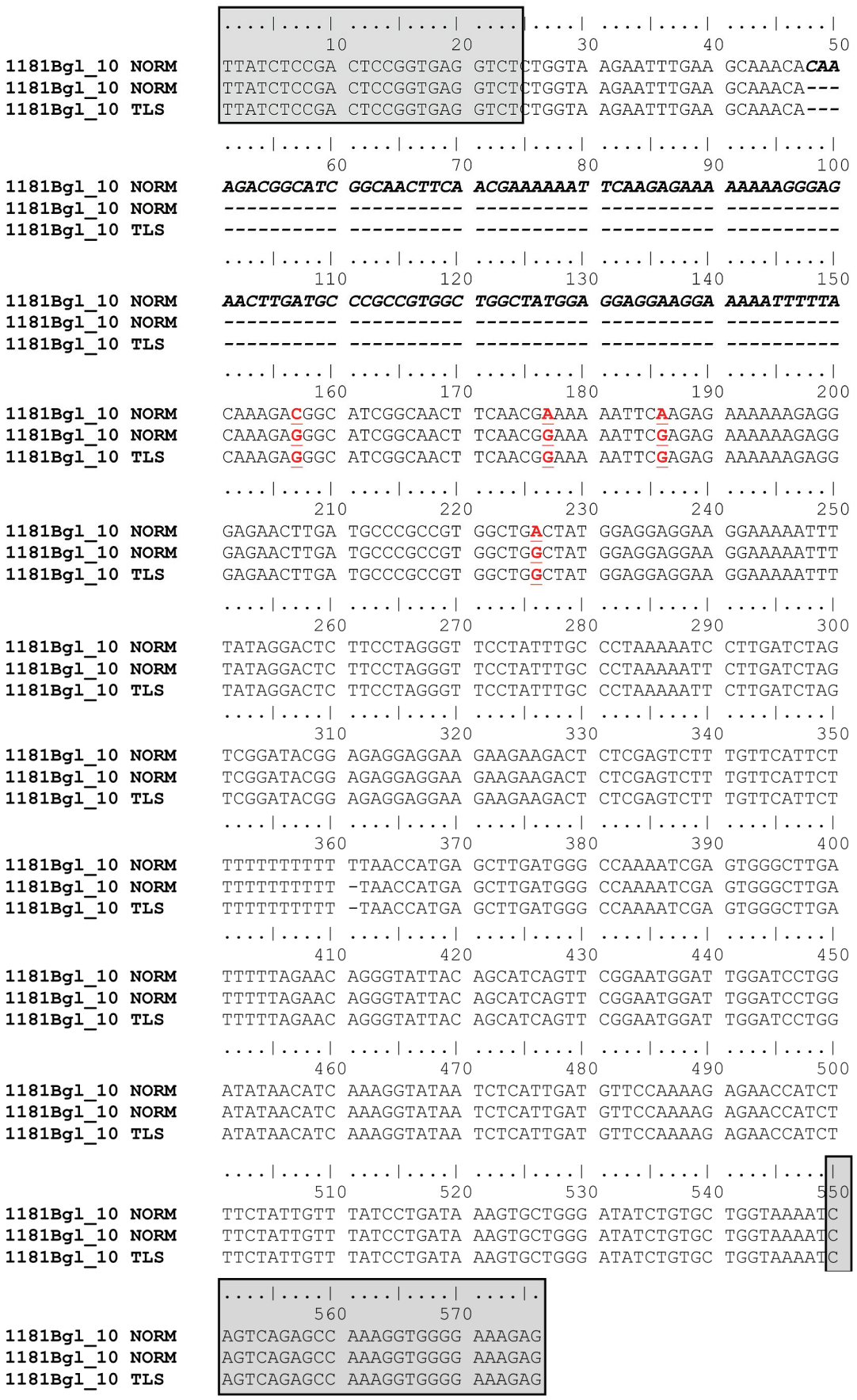

Figure 3. The comparison of nucleotide sequences of the polymerase chain reaction (PCR) amplified products using 1181Bgl (10) primer set and 1181 [normal and Truncated Leaf Syndrome (TLS)] genomic DNA as template. Mismatched nucleotides or different nucleotides are bolded with red font and underlined, presence of extra region is bolded and italicised, while primer sequences are boxed and shaded.

TABLE 5. DETAILS OF THE OIL PALM CLONES USED IN THE VERIFICATION PROCESS THROUGH SOUTHERN BLOT ANALYSIS

Clone No.	Genotype (♀ x ♂)	Classification	
		Abnormal	Normal
1181	Deli *Dura* x Yangambi	Severe	Normal
1077	Deli *Dura* x Dumpy	Severe	Normal
5313	Deli *Dura* x Dumpy	Severe	Normal
5074c	Deli *Dura* x Dumpy	Severe	Normal
4807	Deli *Dura* x Dumpy	Sever	Normal
4874	Deli *Dura* x Dumpy	Severe	Normal
1072	Deli *Dura* x Dumpy	Moderate	Normal
1073	Deli *Dura* x Avros	Moderate	Normal
2818	Deli *Dura* x Dumpy	Moderate	Normal
2751	Deli *Dura* x La Me	Mild	Normal
1096	Deli *Dura* x Dumpy	Mild	Normal
5099	Deli *Dura* x Dumpy	Mild	Normal

Note: ♀ - Female. ♂ - Male.

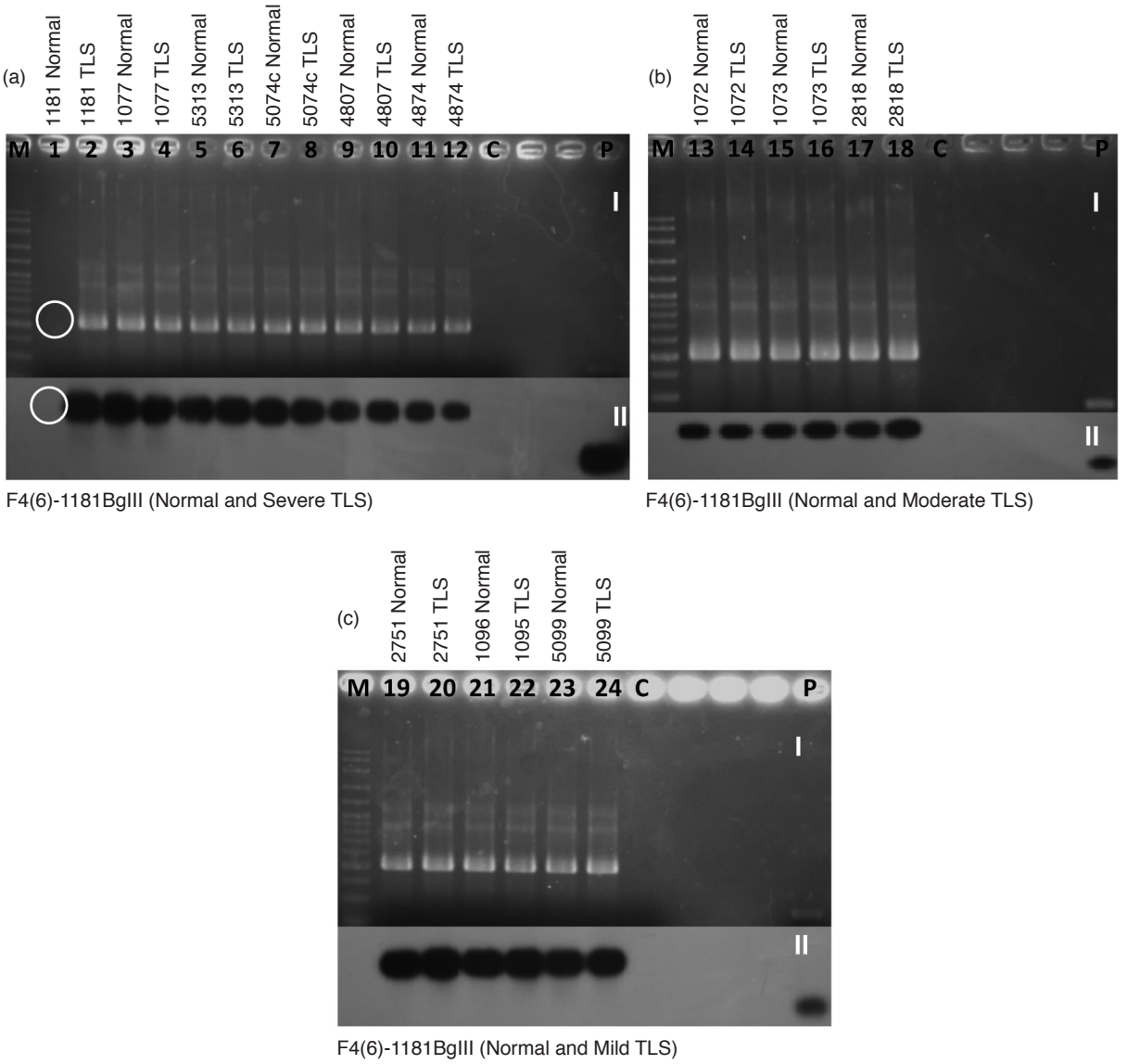

Figure 4 (a, b and c). Polymerase chain reaction (PCR) and Southern analysis of forward Genomic Representational Difference Analysis (G-RDA) using F4(6)-1181BglII primer set. (I) PCR amplified insert DNA from individual oil palm ramets electrophoresed on 2.0% (w/v) agarose gel. (II) Autoradiograph of a Southern analysis result containing the same DNA as shown in (I) that was hybridised with biotin-labelled F4(6)-1181BglII G-RDA difference product. Lane M: 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23: Genomic DNA from normal oil palm ramets (Table 5)*; lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24: Genomic DNA from Truncated Leaf Syndrome (TLS) oil palm ramets* (Table 5)*; lane C: Negative control (without template); lane P: positive control (product of final round of respective G-RDA).*

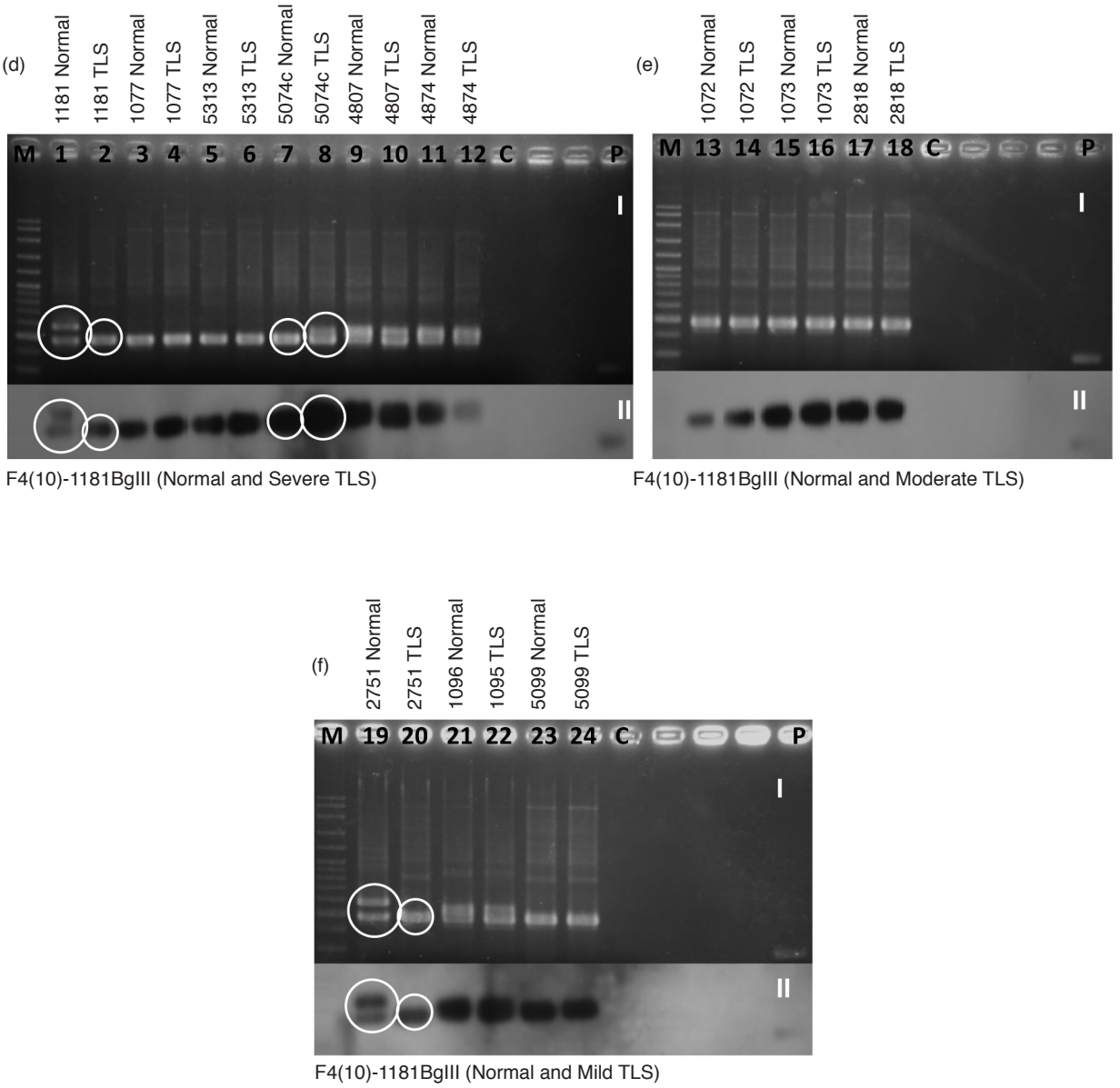

Figure 4 (d, e and f). Polymerase chain reaction (PCR) and Southern analysis of forward Genomic Reprentational Difference Analysis (G-RDA) using F4(10)-1181BglII primer set. (I) PCR amplified insert DNA from individual oil palm ramets electrophoresed on 2.0% (w/v) agarose gel. (II) Autoradiograph of a Southern analysis result containing the same DNA as shown in (I) that was hybridised with biotin-labelled F4(10)-1181BglII G-RDA difference product. Lane M: 100 bp GeneRuler™ DNA ladder (Fermentas, Canada); lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23: genomic DNA from normal oil palm ramets (Table 5)*; lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24: Genomic DNA from TLS oil palm ramets* (Table 5)*; lane C: negative control (without template); lane P: positive control (product of final round of respective G-RDA).*

The clone 5074c showed the opposite result as compared to the clones 1181 and 2751 with primer set F4(10)-1181BglII [lanes 7 and 8 indicated by circle in *Figure 4d* (I and II)] with the presence of single band in normal and double band in TLS ramet. On the other hand, three clones showed the presence of double bands in both normal and TLS ramet (4807, 4874 and 1096) as shown in both gel photograph and X-ray film [lanes 9, 10, 11, 12, 21 and 22 in *Figures 4d* and *4f* (I and II)]. The rest of the clones (1077, 5313, 1072, 1073, 2818 and 5099) showed the presence of single band in both TLS and normal ramet [lanes 3, 4, 5, 6, 13, 14, 15, 16, 17, 18, 23 and 24 in *Figures 4d*, *4e* and *4f* (I and II)].

The presence of complex hybridisation pattern suggests that the hybridised probe detected several bands that could represent various types of repeated sequences and the variation in size would than corresponds to polymorphisms (Zoldos *et al*., 2001). Pluhar *et al*. (2001) reported that the presence of various types of repeated sequences might be due to the genome stress induced in tissue culture

process (Vorster *et al*., 2002). The components in culture media and the type of explants used to generate tissue culture plantlets are some of the inducing factors for somaclonal variation (Leva *et al*., 2012). An imbalanced concentration of growth regulators such as auxins and cytokinins may induce polyploidy in tissue culture regenerants (Swartz, 1991). Endopolyploidy, polyteny and amplification or diminution of DNA sequences are the genome changes that can occur during somatic differentiation in callus culture of plantlets (D'Amato, 1977). Both qualitative and quantitative changes in plant genome can happen during dedifferentiation and redifferentiation process which can cause amplification or deletion of DNA sequences (D'Amato, 1977). Phillips *et al*. (1994) reported that tissue culture process may induce disruption in cellular control that leads to the changes in the number of repeated sequences present in tissue culture regenerants. These changes are most likely affected by mitotic recombination which results in either gain or loss of genetic information.

The banding patterns observed in *Figure 4* (*d*, *e*, and *f*) were not consistent among the tested samples. Hence, PCR products of chosen oil palm clones that showed complex hybridisation pattern (1181, 5074c and 4807) were sequenced and alignment result (*Figure 5*) revealed that those sequences represent multiple regions within the same genome.

Even if there is no consistent difference detected between the normal and TLS genomes, most primer sets were able to differentiate between individual region of normal and TLS oil palm ramet. Since, the oil palm genome is large, 1.8 gigabase (Singh *et al*., 2013); the use of other additional restriction enzymes would provide more genomic subset for successful subtractive hybridisation. Furthermore, a new tester to driver ratio with preferable range combination would be useful to avoid false positive results. Even though there are some modification steps required in G-RDA, it remains a very useful tool for isolating, cloning and characterising the differences between two complex genomes.

```
                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                          10         20         30         40         50
1181   NORMAL (473 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
1181   NORMAL (576 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACACAA
1181   TLS    (473 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
5074c NORMAL (472 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
5074c TLS    (472 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
5074c TLS    (512 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
4807   NORMAL (472 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
4807   NORMAL (512 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
4807   TLS    (472 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---
4807   TLS    (512 bp)   TTATCTCCGA CTCCGGTGAG GTCTCTGGTA AGAATTTGAA GCAAACA---

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                          60         70         80         90        100
1181   NORMAL (473 bp)   ---------- ---------- ---------- ---------- ----------
1181   NORMAL (576 bp)   AGACGGCATC GGCAACTTCA ACGAAAAAAT TCAAGAGAAA AAAAAGGGAG
1181   TLS    (473 bp)   ---------- ---------- ---------- ---------- ----------
5074c NORMAL (472 bp)   ---------- ---------- ---------- ---------- ----------
5074c TLS    (472 bp)   ---------- ---------- ---------- ---------- ----------
5074c TLS    (512 bp)   ---------- ---------- ---------- ---------- ----------
4807   NORMAL (472 bp)   ---------- ---------- ---------- ---------- ----------
4807   NORMAL (512 bp)   ---------- ---------- ---------- ---------- ----------
4807   TLS    (472 bp)   ---------- ---------- ---------- ---------- ----------
4807   TLS    (512 bp)   ---------- ---------- ---------- ---------- ----------

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                         110        120        130        140        150
1181   NORMAL (473 bp)   ---------- ---------- ---------- ---------- ----------
1181   NORMAL (576 bp)   AACTTGATGC CCGCCGTGGC TGGCTATGGA GGAGGAAGGA AAAATTT-TA
1181   TLS    (473 bp)   ---------- ---------- ---------- ---------- ----------
5074c NORMAL (472 bp)   ---------- ---------- ---------- ---------- ----------
5074c TLS    (472 bp)   ---------- ---------- ---------- ---------- ----------
5074c TLS    (512 bp)   ---------- ---------- ---------- ---------- ----------
4807   NORMAL (472 bp)   ---------- ---------- ---------- ---------- ----------
4807   NORMAL (512 bp)   ---------- ---------- ---------- ---------- ----------
4807   TLS    (472 bp)   ---------- ---------- ---------- ---------- ----------
4807   TLS    (512 bp)   ---------- ---------- ---------- ---------- ----------
```

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                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                     160        170        180        190        200
1181   NORMAL (473 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
1181   NORMAL (576 bp)  CAAAGAGGGC ATCGGCAACT TCAACGAAAA AATTCAAGAG AAAAAAGAGG
1181   TLS    (473 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
5074c NORMAL (472 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
5074c TLS    (472 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
5074c TLS    (512 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
4807   NORMAL (472 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
4807   NORMAL (512 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
4807   TLS    (472 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG
4807   TLS    (512 bp)  CAAAGAGGGC ATCGGCAACT TCAACGGAAA AATTCGAGAG AAAAAAGAGG

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                     210        220        230        240        250
1181   NORMAL (473 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
1181   NORMAL (576 bp)  GAGAACTTGA TGCCCGCCGT GGCTGACTAT GGAGGAGGAA GGAAAAATTT
1181   TLS    (473 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
5074c NORMAL (472 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
5074c TLS    (472 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
5074c TLS    (512 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
4807   NORMAL (472 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
4807   NORMAL (512 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
4807   TLS    (472 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT
4807   TLS    (512 bp)  GAGAACTTGA TGCCCGCCGT GGCTGGCTAT GGAGGAGGAA GGAAAAATTT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                     260        270        280        290        300
1181   NORMAL (473 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
1181   NORMAL (576 bp)  TATAAGACTC TTCCCAGGGT TTCTATTTGC CCTAGAAATC CTTGATCTAG
1181   TLS    (473 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
5074c NORMAL (472 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
5074c TLS    (472 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
5074c TLS    (512 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
4807   NORMAL (472 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
4807   NORMAL (512 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
4807   TLS    (472 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG
4807   TLS    (512 bp)  TATAGGACTC TTCCTAGGGT TCCTATTTGC CCTAAAAATT CTTGATCTAG

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                     310        320        330        340        350
1181   NORMAL (473 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
1181   NORMAL (576 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
1181   TLS    (473 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
5074c NORMAL (472 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
5074c TLS    (472 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
5074c TLS    (512 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA***TCTAG TCGGATACGG***
4807   NORMAL (472 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
4807   NORMAL (512 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA***TCTAG TCGGATACGG***
4807   TLS    (472 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA----- ----------
4807   TLS    (512 bp)  TCGGATACGG AGAGGAGGAA GAAGAAGACT CTCGA***TCTAG TCGGATACGG***

                           ....|....| ....|....| ....|....| ....|....| ....|....|
                                360        370        380        390        400
1181   NORMAL (473 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
1181   NORMAL (576 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
1181   TLS    (473 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
5074c NORMAL (472 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
5074c TLS    (472 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
5074c TLS    (512 bp)  ***AGAGGAGGAA GAAGAAGACT CTCGA***GTCTT TGTTCATTCT TTTTTTTTTT
4807   NORMAL (472 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
4807   NORMAL (512 bp)  ***AGAGGAGGAA GAAGAAGACT CTCGA***GTCTT TGTTCATTCT TTTTTTTTTT
4807   TLS    (472 bp)  ---------- ---------- -----GTCTT TGTTCATTCT TTTTTTTTTT
4807   TLS    (512 bp)  ***AGAGGAGGAA GAAGAAGACT CTCGA***GTCTT TGTTCATTCT TTTTTTTTTT
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                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                        410        420        430        440        450
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1181  NORMAL (576 bp)   TTAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
1181  TLS    (473 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
5074c NORMAL (472 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
5074c TLS    (472 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
5074c TLS    (512 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
4807  NORMAL (472 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
4807  NORMAL (512 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
4807  TLS    (472 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC
4807  TLS    (512 bp)   -TAACCATGA GCTTGATGGG CCAAAATCGA GTGGGCTTGA TTTTTAGAAC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                        460        470        480        490        500
1181  NORMAL (473 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
1181  NORMAL (576 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
1181  TLS    (473 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
5074c NORMAL (472 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
5074c TLS    (472 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
5074c TLS    (512 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
4807  NORMAL (472 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
4807  NORMAL (512 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
4807  TLS    (472 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC
4807  TLS    (512 bp)   AGGGTATTAC AGCATCAGTT CGGAATGGAT TGGATCCTGG ATATAACATC

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                        510        520        530        540        550
1181  NORMAL (473 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
1181  NORMAL (576 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
1181  TLS    (473 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
5074c NORMAL (472 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
5074c TLS    (472 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
5074c TLS    (512 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
4807  NORMAL (472 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
4807  NORMAL (512 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
4807  TLS    (472 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT
4807  TLS    (512 bp)   AAAGGTATAA TCTCATTGAT GTTCCAAAAG AGAACCATCT TTCTATTGTT

                                ....|....| ....|....| ....|....| ....|....| ....|....|
                                        560        570        580        590        600
1181  NORMAL (473 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
1181  NORMAL (576 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
1181  TLS    (473 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
5074c NORMAL (472 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
5074c TLS    (472 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
5074c TLS    (512 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
4807  NORMAL (472 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
4807  NORMAL (512 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
4807  TLS    (472 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC
4807  TLS    (512 bp)   TATCCTGATA AAGTGCTGGG ATATCTGTGC TGGTAAAATC AGTCAGAGCC

                                ....|....| ....|.
                                        610
1181  NORMAL (473 bp)   AAAGGTGGGG AAAGAG
1181  NORMAL (576 bp)   AAAGGTGGGG AAAGAG
1181  TLS    (473 bp)   AAAGGTGGGG AAAGAG
5074c NORMAL (472 bp)   AAAGGTGGGG AAAGAG
5074c TLS    (472 bp)   AAAGGTGGGG AAAGAG
5074c TLS    (512 bp)   AAAGGTGGGG AAAGAG
4807  NORMAL (472 bp)   AAAGGTGGGG AAAGAG
4807  NORMAL (512 bp)   AAAGGTGGGG AAAGAG
4807  TLS    (472 bp)   AAAGGTGGGG AAAGAG
4807  TLS    (512 bp)   AAAGGTGGGG AAAGAA
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Figure 5. The comparison of nucleotide sequences of the polymerase chain reaction (PCR) products amplified using the F4(10)-1181BglII primer set. Genomic DNA from clones 1181 [normal and Truncated Leaf Syndrome (TLS)] , 5074c (normal and TLS) and 4807 [normal and were used as template. Presence of extra region is bolded and italicised, while primer sequences are boxed and shaded.

CONCLUSION

The subtractive hybridisation method (G-RDA) was used in this study to reveal the genomic differences present in normal and TLS oil palm ramet. A few different products were successfully cloned and characterised. The obtained data suggests that the differences between the normal and TLS genome are due to single nucleotide substitution and an insertion or a deletion of a portion of sequences in the normal and TLS genomes. Further verification using the oil palm clones from the same variety is needed to elucidate the ability of the two sets of primer pair [F4(6)-1181BglII and F4(10)-1181BglII] as a candidate biomarker to identify TLS plantlets during tissue culture process.

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