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Identification of Gene Related to Hard Bunch Phenotype in Oil Palm (*Elaeis guineensis* Jacq.)

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ABSTRACT

Molecular genetic analysis of hard bunch phenomenon in oil palm was done in order to elucidate the role of genetic factor underlying hard bunch in oil palm plantation. The aim of this study was to identify the AFLP primer combination that co-segregates with hard bunch phenotype related gene in oil palm. Molecular analysis was done by bulk segregant analysis approach. DNA was isolated from leaves of the normal and hard bunch palm. DNA from ten individual palms from each category were pooled and used as a template. A total of 56 AFLP primer combinations were selected for selection of polymorphic primer, and as a result it was found that 22 AFLP primer combinations (39.28%) were polymorphic. A total of 48 individual of palm DNA containing 24 individual for each group were further genotyped by those 22 polymorphic markers. Of these, one AFLP primer combination (E-ACC/M-CTG) was obtained as a co-segregated marker that distinguished the hard bunch DNA from the normal one. Based on the analysis of the target sequence aligned to the oil palm DNA sequences available in database, we found that our sequence has similarity with Ty-1 copia retrotransposon. This sequence distribute in all 16 linkage group of oil palm genome.

Keywords: abnormal fruits, AFLP, oil palm, Ty-1 copia retrotransposon

ABSTRAK

Analisis molekuler terhadap fenomena tandan buah keras dilakukan dalam rangka mengetahui peranan faktor genetik dalam fenomena tersebut di lapang. Tujuan dari penelitian ini adalah untuk mendapatkan marka AFLP yang terkait dengan fenomena tandan buah keras. Analisis molekuler dilakukan dengan pendekatan bulk segregant analysis. DNA diisolasi dari daun tanaman yang mempunyai buah normal dan tandan buah keras. DNA dari sepuluh tanaman dari tiap kelompok digabungkan dengan jumlah dan konsentrasi yang sama untuk digunakan sebagai cetakan dalam proses PCR. Sebanyak 56 kombinasi primer yang mengamplifikasi DNA genom kelapa sawit digunakan dalam seleksi primer polimorfik. Hasil seleksi didapatkan sebanyak 22 kombinasi primer (39.28%) polimorfik. Sebanyak 48 DNA tanaman yang terdiri dari 24 individu masing-masing kelompok diamplifikasi menggunakan primer polimorfik. Satu kombinasi primer yaitu E-ACC/M-CTG secara konsisten muncul pada DNA tanaman tandan buah keras dan tidak ada pita pada DNA tanaman buah normal. Hasil analisis memperoleh sekuen pita spesifik mempunyai kemiripan dengan Ty-1 copia retrotransposon. Sekuen tersebut terdistribusi secara merata pada 16 kelompok pautan genom tanaman kelapa sawit.

Kata kunci: AFLP, Buah abnormal, Ty-1 copia retrotransposon

INTRODUCTION

Hard bunch is the non-shed ripe fruits and some fruits remain attached to the bunch after the process of separating the fruit from bunch at the thresher of the mill. This phenomenon is uncommon in oil palm plantation and only occurs in the area that has consecutive dry periods during the year. This phenomenon results in a loss in the estate or in the oil palm factory.

The phenomena of hard bunch are unstable and unique. The palms which bear hard bunch fruits could produce normal fruits and growing next to palms that always produce the normal bunches. There is no report for this phenomenon so far. The main factor causes this phenomenon is not well understood, whether it is affected by genetics, environment or interaction of both factors. In order to study the effect of genetics factor involved in this phenomenon, the characterization of genomic variation in molecular marker level is needed.

Recent progress in plant genomics analysis has allowed the geneticist and plant breeders to identify genes for plant improvement. Numerous kinds of molecular markers are now available such as *Simple Sequence Repeat* (SSR), *Amplified Fragment Length Polymorphism* (AFLP), and

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Single Nucleotide Polymorphism (SNP). Of these, AFLP is one of the best markers for plant diversity analysis (Yang *et al.,* 2005) and it is widely used because of its ability in revealing diversity at the species level and provide an effective means of covering a wide area of the genome in a single assay.

The other advantages of using AFLP marker is that the specific band could be converted to SCAR marker (Sobir *et al.,* 2008). Polymorphic DNA could be directly associated to phenotypic differences, genetically linkage to regulatory factor or indicated relatedness of individual among population (Jhanwar *et al.,* 2012).

AFLP markers combined with *Bulk Segregant Analysis* (BSA) method have been widely used to discover marker closely associated with trait in many crops including *Brassica napus* (Zeng *et al.,* 2009) and tomato (Miao *et al.,* 2009). AFLP markers have been used for analysis of oil palm genome (Barcelos *et al.,* 2002), date palm genome (Rhouma *et al*., 2007) as well as other crops such as *Amorphophallus variabilis* (Santosa *et al.,* 2012).

Based on the above advantages, AFLP markers combined with BSA methods were used in this study. BSA was used because there are two different populations in the plantation. One population is the palms bearing hard bunch and the other is the palms that are always producing normal bunches. The objectives of this study were to identify the AFLP primer combination which could distinguish between hard bunch and normal palms and to get the information of sequence similarity specific with known genes in the database. Markers obtained is expected to be a selection tool for parental crossing candidate.

MATERIALS AND METHODS

Genetic Materials

A total of 48 individual oil palm containing 24 palms of each categories (hard and normal bunches) were selected. All palm samples are of the same progenies but have different phenotypic performance. Samples were taken from Lampung Region, on 20 years Tenera (D x P) palm. The groups for normal bunch palms are palms which have been identified always produce normal bunches and the other groups for hard bunch are palms that bearing hard bunches. Leaf samples from normal and hard bunch palms were collected and put it in the plastic bag and labelled. The samples were put in cooler box with dry ice and sent to the laboratory for further processing. A total of 64 AFLP markers which synthesized by Invitrogen (USA) were used in genotyping analysis. The research was conducted during April 2012 to September 2014.

DNA Isolation

DNA was isolated from oil palm leaf using *Nucleospin Plant II kit* (Macherey-Nagel, Germany), according to manual instruction. The quality and quantity of extracted DNA were measured by Spectrophotometer Nanodrop 2000C (Thermo Scientific, USA).

Bulked Segregant Analysis (BSA) with AFLPs

BSA with AFLP markers was used to identify markers co-segregate with hard bunch palms. The DNA pools were prepared by combining equal amount of the aliquots of DNA from ten individuals that were identical for particular traits (hard bunch and normal palms). The AFLP procedure was performed as described by Vos *et al*. (1995) which comprised of three steps, as follows.

Template DNA preparation

Approximately 250 ng of genomic DNAs of each bulk were digested for 2 h at 37 °C with 1.25 U μL^{-1} of an *Eco*RI/*Mse*I mixture (each in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg mL^{-1} BSA, 50% (v/v) glycerol, 0.1% Triton X-100) and 5 µL of a 5X reaction buffer (50 mM Tris-HCI (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate) in a total volume reaction of 25 µL. After digestion, the digested DNAs were ligated to adaptor/ligation solution (*Eco*RI/*Mse*I adaptor, 0.4 mM ATP, 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) using 1 U μL^{-1} T4 DNA ligase (Gibco BRL). The mixture was incubated for 2 h at 20 °C for ligation and the mixture was then diluted ten times with deionised water as template DNA and stored at 4 °C for being used in further steps.

Pre-amplification

After adaptor ligation, pre-amplification of DNA fragments was performed as described in Vos *et al*. (1995) in a total reaction of 51 µL as follows: 5 µL of ligated DNA, 40 µL pre-amp primer mix, 5 µL 10X PCR buffer plus Mg and 1 µL of 0.5 U Taq polymerase (Promega, WI, USA). Samples were run for 20 cycles of 94 °C (30 s), 56 °C (1 min), and 72 °C (1 min). Pre-amplification products were then diluted 50X in double-distilled H_2O, and used as templates for selective amplification.

Selective Amplification

Selective amplification of the pre-amplified DNA was carried out using 64 AFLP primer combinations, with 3-base-pair extension in a 20 µL reaction containing: 5 µL diluted pre-amplification product, 4.5 µL selective *Mse*I primer (contains dNTPS) and 0.5 µL selective *Eco*RI primer, 2 µL of 10X PCR buffer, 1 µL of 5 U Taq polymerase (Promega) and 7.9 µL distilled water. The following cycle profile ensured optimal selective amplification: one cycle of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C, followed by 12 cycles of 0.7 °C lower annealing temperature for each cycle, and 23 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C.

Electrophoresis

Amplification products were separated on a 6% polyacrylamide (acrylamide: bisacrylamide = 19:1) /7.5

M urea in 1X TBE buffer (0.09 M Tris-borate and 0.002 M EDTA), and detected by silver staining according to procedure described in Benbouza *et al.* (2006). Band sizes were determined by comparison presence bands with a 1Kb plus DNA ladder size standard from Gibco-BRL (MD, USA).

Polymorphic Primer Verification and Gel Extraction

Polymorphic primers were used for amplifying all of 24 individual sample of each bulk to see if the band consistently appears in each group. Consistent band were cut from the gel and the DNA were extracted with *Qiaquick Gel Extraction Kit* (Qiagen, Germany), according to manufacturer instruction. DNA concentration and purity was checked by spectrophotometer Nanodrop 2000C (Thermo Scientific, USA).

Amplification and Purification of Specific DNA

DNA specific was amplified using the same primer combination with the following cycle profile included 25 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C on the 100 µL total reaction. Amplification product or amplicon was mixed with 3 µL of 6X loading dye and checked by electrophoresis in a 1% agarose gel. Amplicon with showed only one band was selected and purified with *QIAquick PCR purification kit* (Qiagen, Germany), according to manufacturer instruction. Concentration of the DNA was checked by the same procedure as previously described above.

Sequencing and Data Analysis

Purified DNA samples were sent to Genetica Science, Singapore. Sequence was analyzed using BLAST software (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.,*1990) and local database.

Primer Design, PCR and Sequencing

Sequence of specific DNA was used to primer design by Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) software. DNA of hard bunch and normal palm were amplified with designed primer. PCR composition consists of 5 µL of 5X KAFA2G buffer A, 0.5 µL of 20 mM dNTP mix, 1.25 µL of 10 mM each primer, 0.10 µL of 5 U μL^{-1} KAFA2Fast DNA Polymerase, 14.90 µL water and 2.0 µL DNA. PCR program was done as follow: at 95 ^{o}C for 3 min, 40 cycles at 95 ^{o}C for 10 s, at 62 ^{o}C for 10 s, at 72 ^{o}C for 5 s, and final extension at 72 ^{o}C for 3 min. Amplified product was cut from the gel, and treated with the same procedure described above.

RESULTS AND DISCUSSION

Based on genotyping analysis of 64 AFLP primer combinations screened in oil palm DNA samples provided clear and unclear banding patterns. In the other words, the study successfully identified that some of the AFLP primer combinations could not provide good band. Moreover, 2 out of 64 AFLP primer combinations were M-CTG and M-CTT could not amplify oil palm genome, whereas 6 primers amplified the DNA genomics with less clear band. On the other hand, a total of 56 primer combinations successfully provided amplicons showing good band patterns (Figure 1). The selective amplification was achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites (Vos *et al.,* 1995). We also investigate that AFLP primer combinations which could not amplify oil palm genome were generally derived from the combination of M-CTG. In present study, however, only combination of M-CTG/E-ACC could amplify oil palm genome (Table 1).

The 56 AFLP primer combinations which resulted good band were used for selection of polymorphic primers. Of these, 22 primers (39.28%) were found as polymorphic markers between hard bunch and normal bunch. The number of polymorphic primers found in present study is higher than those found in previous study as reported by Mathius *et al.* (2005) that found 10 polymorphic primers between normal and mantled palm derived from tissue culture.

AFLP combined with BSA approach has successfully been used in identifying molecular markers associated with crown rust resistance in perennial ryegrass (Muylle *et al.,* 2005) and detection of variation of oil palm tissue culture ramet (Lei *et al.,* 2006). This method was also applied to identify molecular markers that associated with resistance to bacteria in tomato (Miao *et al.,* 2009), and those associated to cytoplasmic male sterility in *Brassica napus* (Zeng *et al.,* 2009).

The total 22 detected polymorphic primer combinations found in present study were used for amplifying 48 individual plant, which contained 24 individuals DNA samples per each group (normal and hard bunches palm). Of these, one primer combination of E-ACC/M-CTG was consistently appeared in hard bunch palm and was absent in normal bunch which detected in the position of 200 bp. While the other primer combinations showed inconsistentcy band for each group. Hence, among tested AFLP combination primers, E-ACC/M-CTG was the only co-segregated marker with hard bunch trait that would be useful to identify gene involved in hard bunch phenomenon in oil palm.

Based on analysis of specific band sequence amplified by E-ACC/M-CTG primer in hard bunch DNA that aligned to the predicted gene in database, the target sequence showed a high similarity with Ty-1 copia retrotransposon. It is known that the oil palm genome has a high portion of repeated sequence. One of these is Copia-like retrotransposon which is distributed over all oil palm genome (Castilho *et al.,* 2000). Transposon could be activated by biotic and abiotic stress (Zeh *et al.,* 2009). Present result indicated that the transposon was activated in oil palm genome due to dry periods exist during the year. Retrotransposon activity can be a major factor in genome instability and rearrangements

and therefore also increase plasticity of the genome and adaptation to changing environmental conditions (Voronova *et al.,* 2012).

Transposon contributes and involves in genomic evolution, genome structure and gene function. The transposon activation could cause mutation so it is a risk for plant. Transposon element exists in all species, but its effects to the genome varies although the plant is closely related. This evidence occurs by their activity such as transposition, insertion and chromosome breakage (Yu *et al.,* 2010).

Sequence of specific product was used as a template for design new primer. The amplicon of this new primer was a partial for Ty-1 copia retrotransposon. Analysis of sequence amplified by new designed primer showed that the sequence has high similarity with Ty-1 copia retrotransposon. This sequence distributed along 16 linkage groups of oil palm genome which published by Singh *et al.* (2013), available at http://www.genomsawit.mpob.gov.my. (Table 2).

Ty-1 Copia retrotransposon belongs to long terminal repeat (LTR) retrotransposon and reported as the most abundant elements in plant genomes and non-autonomous element (Wicker *et al.,* 2007). Because of their replicative mode of transposition based on an RNA intermediate, they compose the majority of the DNA of many eukaryotic genomes. They are particularly abundant in plant genomes and are intimately involved in the evolution of genome structure and size (Estep *et al.,* 2013)

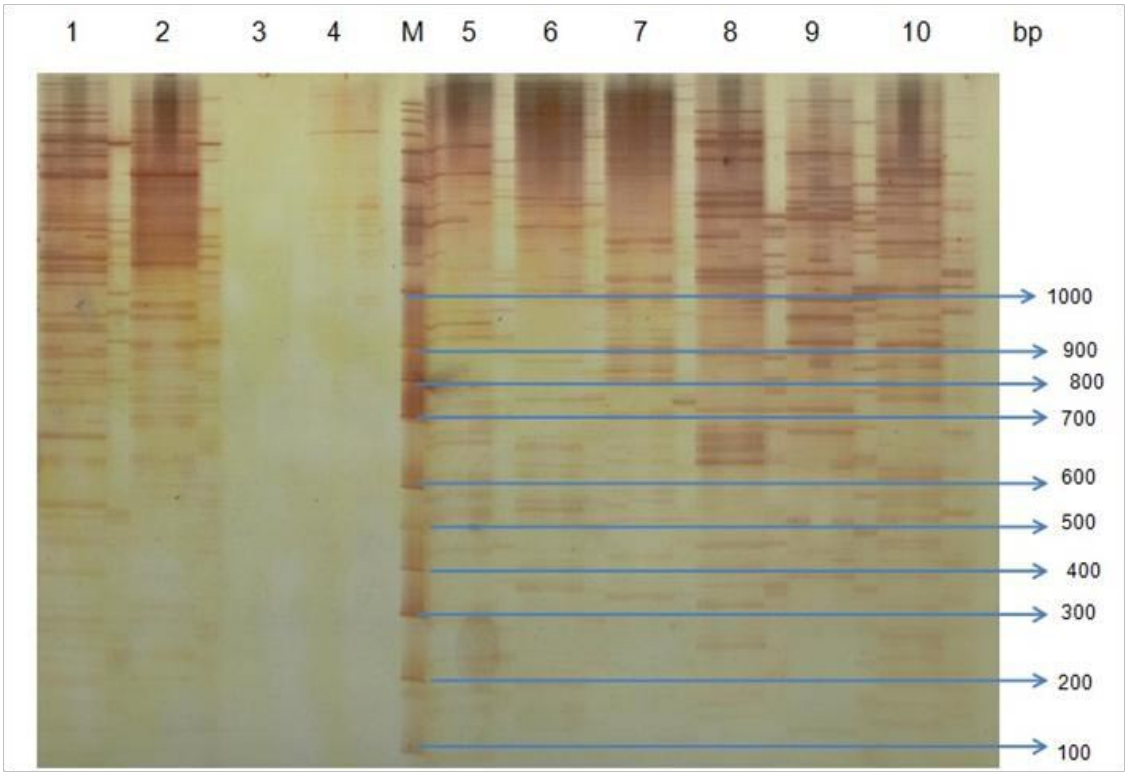

Figure 1. DNA profiles of oil palm genotyped by some AFLP primer combinations provided good (no 1,2, 5-10) and without band patterns (no 3 and 4) M = 1 kb plus DNA ladder

Table 1. AFLP primer combinations profiles on oil palm genomics DNA

Primer combination	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√	√	√	√	√	-	-
E-AAG	√	√	√	√	√	√	-	√
E-ACA	√	√	√	√	√	√	-	√
E-ACC	√	√	√	√	√	√	√	√
E-ACG	√	√	√	√	√	√	-	√
E-ACT	√	√	√	√	√	√	-	√
E-AGC	√	√	√	√	√	√	-	√
E-AGG	√	√	√	√	√	√	-	√

√ indicated combination of AFLP primer provided amplification products in oil palm DNA genome; '-' no amplification

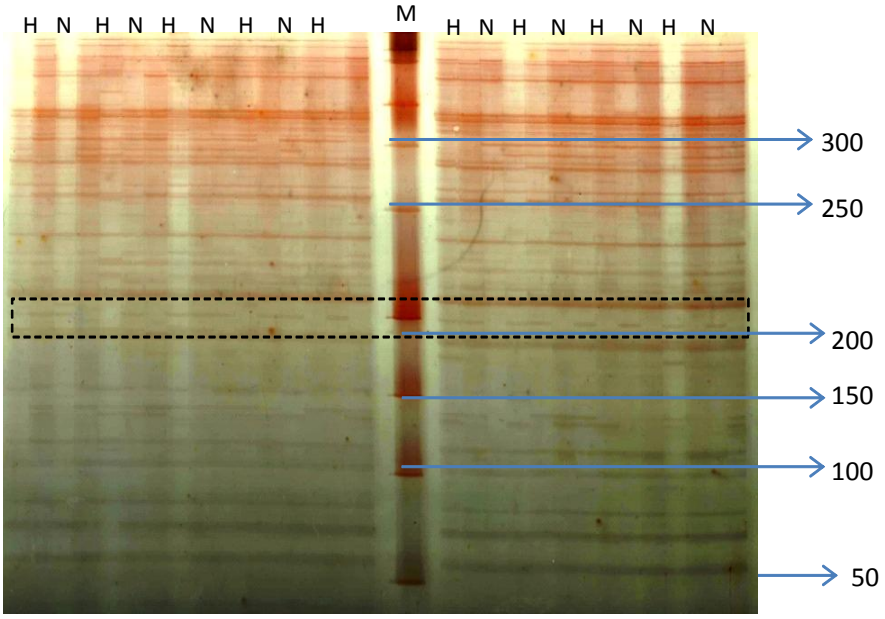

Figure 2. Banding pattern resulted from individual DNA samples using primer E-ACC/M-CTG. H = hard bunch, N= normal bunch and M = Marker 100 bp DNA ladder

Table 2. Sequence similarity of amplicon with oil palm genome

Linkage group	Similarity	Persentage
1	352 / 387	90
2	341 / 388	87
3	138 / 150	92
4	348 / 385	90
5	347 / 381	91
6	350 / 385	90
7	344 / 381	90
8	341 / 387	88
9	322 / 361	89
10	339 / 377	89
11	347 / 380	90
12	344 / 388	88
13	350 / 387	90
14	212 / 230	92
15	354 / 387	91
16	342 / 381	89

CONCLUSION

This study successfully identified and mapped an AFLP primer combination of E-ACC/M-CTG as a locus that co-segregates with hard bunch phenotype related gene in oil palm which specifically distinguished the hard bunch from the normal one. Analysis of the target sequence of the specific band showed a high similarity with Ty-1 copia retrotransposon distribute in all 16 LG oil palm genome.

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