

REGULAR ARTICLE

Differential analysis of gene related to hard bunch phenomena in oil palm (*Elaeis guineensis* Jacq) fruits

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ABSTRACT

Identification of differentially expressed genes can be done by cDNA-AFLP analysis. Hard bunch phenomena are not widely studied. The aim of this research was to identify and characterize regions specific to the gene coding sequences involved in the hard bunch phenomena in oil palm fruits. RNA was isolated from three types of ripe oil palm fruits including normal, black and hard bunch fruits. cDNAs were amplified using 16 AFLP primer pairs combinations. The result of a BLAST comparison showed that some of the resulting transcripts have sequence similarity with the existing database sequences, such as a WRKY transcription factor, peroxidase, actin depolymerising factor and glutamine synthetase allegedly associated with hard bunch phenomenon induced by drought stress. However, some transcripts could not be classified since they did not exhibit significant similarity to any existing database sequences. This might be the transcripts were less conserve region and or 5' or 3' UTR region.

Keywords: Hard bunch; Oil palm; Peroxidase enzyme; WRKY transcription factor

INTRODUCTION

Hard bunch is the non-shed ripe oil palm fruits and some fruits remain attached to the bunch after the process of separating the fruit from the bunch at the thresher of the mill. Hard bunch phenomena causes two problems in oil palm industry. First, many bunches harvested late because there are no losses of fruit, as bunch harvest indicator in the plantation, resulting in lower productivity of the plantation. Secondly, hard bunch caused reducing oil extraction ratio in oil palm plantations and low *Crude Palm Oil* (CPO) quality do to high of *Free Fatty Acid* (FFA) content.

This phenomenon occurred when the plants were exposed to water deficit severely during six to nine months before harvesting fruit bunches. Water deficit was triggered by low rainfall and supported by other climatic elements that cause the increase of the plant transpiration rate, such as the relative humidity, the wind speed and the distribution of sunshine hours (Roberdi, 2014). The phenomena are reported occurs in Southern of Sumatera. Water deficit combined with mechanical stress can reduced sex ratio in oil palm (Ajambang et al., 2015).

Oil palm requires ± 2000 mm of rainfall evenly distributed throughout the year. Therefore, hard bunch phenomenon of the oil palm fruits produced in the area that meets this requirement phenomenon is rarely observed. The study about biochemical differences in two type of bunches, AFLP analysis of the genome and climatic factors related to this phenomena were studied (Roberdi, 2014) and the biochemical pathway involved in the hard bunch phenomenon, especially at molecular level were studied as well.

Identification and investigation of gene expression patterns in a particular stage of both plant growth and development is important step in elucidating the function of genes, metabolic processes and plant responses to environmental conditions. Gene expression in plants occurs through accurate regulatory process, including coordination between the genes in the nucleus and between genes in nucleus and organelles (Woodson and Chory, 2008). Some of genes are expressed in tissues and organs at certain developmental stages. Genes involved in important traits can be determined by making a cDNA library of the various phases of the development of plant tissues and organs.

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Afterwards, cDNA library can be sequenced to generate an *expressed sequence tags* (EST) database that can be utilized in new genes discovery, confirmation of the sequences in the genome, elucidation of phylogenetic relationships, construction of genomic maps, comparative genomic studies, and used as the basis for the analysis of gene expression (Fei et al., 2004; Alba et al., 2004). Recently, oil palm ESTs have been analyzed for the gene expression in tissue culture process, comparative genomic of oil palm with other plants, analysis of metabolites in the oil palm mesocarp, and gene expression in several organs (Bourgis et al., 2011; Tranbarger et al., 2012).

In respect to gene discovery related to the phenomena of the hard bunch fruits, the cDNA libraries using AFLP marker or called as cDNA-AFLP (Bachem et al., 1996) can be potentially used for differential expression analysis. In comparison to other RNA analysis techniques such as differential display technique, cDNA-AFLP technique provides several advantages, such as no sequence information of the plant genomes is required, the initial cost is not too expensive, and high specificity products. This technique is reliable, generating a lot of bands, making it possible to identify rare transcripts. With these advantages, it is a great opportunity to construct a comprehensive EST database by sequencing the entire fragment specific.

cDNA-AFLP technique has been done for the differential analysis of apple fruit (Yao et al., 2007). However, the gene involved in the hard bunch fruits phenomenon is not well studied. Therefore, the cDNA-AFLP approach can also be applied in studying the genes involved in the hard bunch fruit of oil palm. In order to obtain the information of genes involved in oil palm fruit ripening and detaching fruit, it is necessary to analyze the sequences from both hard bunch and normal fruits. The aim of this research was to identify and characterize a differential expressed genes involved in hard bunch phenomena in oil palm fruits

MATERIALS AND METHODS

Genetic material

Ripe normal, hard bunch and black fruits were harvested from 20 years of Tenera (D x P) palm Deli origins. Fresh mesocarp were cut and put into the 2 mL microtube which already contains RNAlater (Ambion, USA). Samples were sent to the laboratory and stored at -20°C before use.

RNA isolation and quantifications

Total RNA Mini Kit Plant (Geneaid, USA) was used for RNA isolation according to the manufacturer instruction. RNA purity and quality was checked using

Spectrophotometer Nanodrop 2000C (Thermo Scientific, USA), and QIAxcel (Qiagen, Germany).

cDNA synthesis

SMARTER PCR cDNA Synthesis Kit (Clontech, USA) was used for cDNA synthesis according to manufacturer instruction. PCR condition is denaturation at 95°C for 60 s, continued for 27 cycles with denaturation at 95°C for 15 s, primer annealing at 65°C for 30 s and extension at 72°C for 3 min.

Template preparation, AFLP PCR and electrophoresis

PCR template was prepared based on protocol described by Bachem et al. (1996). cDNA was digested with *EcoRI* and *MseI* for 2 h at 37°C, and ligated to adaptor according to *Core AFLP kit* (Gibco-BRL) procedure. PCR was done for 20 cycles with denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min and extension at 72°C for 1 min, using primer which correspond to adaptor without adding any specific Nitrogen base.

Pre-amplification product was diluted 10 times with TE buffer. Five micro litres were used for selective amplification. Selective amplification consisted of 23 cycles, 13 cycle touchdown from 65°C to 56°C, with decreased 0.7°C each cycles. Further conditions followed by 15 cycles of PCR. A total of 16 AFLP primer combinations were used for selective amplification. The primer combinations such as *EcoRI*-AAC, ACC, ACG and *MseI*-CTT, CAT, CTA, CAC, and CTG were used in this study. Products of selective amplification were electrophoretically separated in a 6% polyacrylamid gel and the staining process was based on procedure described by Benbouza et al. (2006).

Isolation of transcript-derived fragment (TDF)

TDFs were cut from the gel and purified with *Qiaquick Gel Extraction Kit* (Qiagen, Germany) according to manufacturer instruction. Purified DNA was amplified using same primer combination with selective amplifications for 25 cycles. PCR profile was carried out at the following steps including denaturation at 94°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min. PCR Products were purified using *Qiaquick PCR Purification Kit* (Qiagen, Germany) according to the manufacturer instruction.

Sequence analysis

Purified DNA was sent to sequencing provider. Sequencing results were aligned to database with BLAST from NCBI (www.ncbi.nlm.nih.gov) and local database. Each TDF was aligned using blastn and blastx (Altschul et al., 1990). Sequences which showed similarity were analysed with Blast2GO software (Conesa et al., 2005) and *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (Kanehisa and Goto, 2000).

RESULT AND DISCUSSION

We obtained around 20 specific bands generated by 16 AFLP primer combinations for each type of fruits. Each AFLP primer combination produced different specific bands for fruit types (Fig. 1). Length of the amplicons varied between 167-660 bp (Table 1). Specific band will provide information of genes expressed in ripe stage of normal, hard bunch and black bunch fruits. Information obtained would be expected to provide biochemical pathway involved in ripening and fruit losses. Nine specific bands were eluted from the gel and the DNA was extracted.

Based on comparative sequences analysis, four sequences showed similarity with the sequence existed in the public database, whereas five sequences did not show any similarity. Sequences that showed similarity with those in database consisted of WRKY transcription factor, peroxidase enzyme, actin depolymerising factor and glutamine synthetase enzyme (Table 1). Of these, two

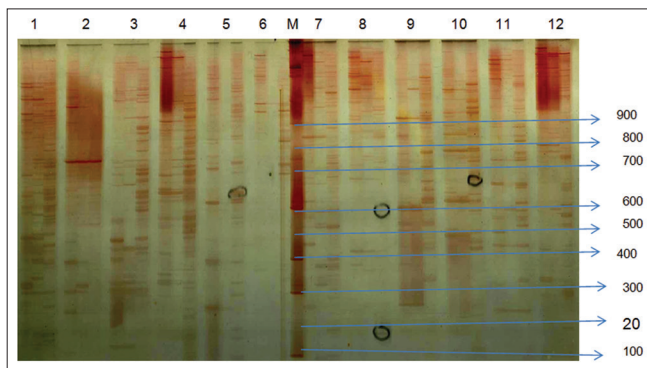


Fig 1. cDNA-AFLP profile from some AFLP primer combinations. Notes: Number 1 to 6 are primer combination E-AAC/M-CTT, E-ACC/M-CAT, E-ACG/M-CTA, E-AAC/M-CAC, E-ACC/M-CTG, E-ACG/M-CTT, respectively. M: Marker DNA ladder 100 bp. Number 7 to 12 are combination primer E-AAC/M-CAT, E-ACC/M-CTA, E-ACG/M-CAC, E-AAC/M-CTG, E-ACC/M-CTT, E-ACG/M-CAT, respectively.

sequences, peroxidase enzyme and glutamine synthetase were already registered which have coding enzyme and their function was also known for various plants according to comparative analysis results in KEGG (www.genome.jp/kegg/).

A large number of sequences that do not have a similarity are might be the sequence obtains are less conserve region and or 5' or 3' Untranslated region of a functional genes. Although not all sequences showing similarity with the sequence existed in the gene bank, their position in oil palm genome can be determined based on sequence published by Singh et al. (2013) at <http://www.genomsawit.mpob.gov.my>. Therefore, we were able to anchor the sequences that have no similarity with any database, such as P1, P2, P3, P4 and P29 were located in the oil palm linkage groups (LG) 1 and 10 (Table 1). The non identity sequences were extend their genome position in order to obtain the near genes. In accordance, the Actin depolymerising factor was located in LG 5 and 14, peroxidase enzyme was in LG 8, glutamine synthetase was in LG 16 and WRKY transcription factor was in LG 10.

The use of genomic data is an important aspect to in investigating the association of the obtained sequences with a specific biological function through functional annotation of sequence. Functional annotation would classify genes into specific functional classes. A gene information product will be helpful in interpreting the research data. Analysis of the sequences using Blast2GO software could help us to determine the molecular function of sequences involved in various biological or cellular processes. Therefore, the information of molecular function and their cellular locations of these sequences involved in various cellular processes was also collected and presented in Table 2.

In general, plants have two classes of peroxidases (PODs) comprised of peroxidase I and peroxidase III. Class I

Table 1: Sequence specific analysis from cDNA-AFLP

| Sequence | LG | Bp | Similarity | Neighbour gene | Position | Pathway |
|----------|-------|-----|--|--------------------------------------|-------------------|--------------------------------------|
| >P1 | 10 | 210 | No similarity sequence | Thioredoxin like | 2.7 kb up stream | |
| >P2 | 10 | 264 | No similarity sequence | ATP dependent RNA helicase dhx8-like | 283 bp upstream | |
| >P3 | 10 | 212 | No similarity sequence | Ygz7 uncharacterized mRNA | 342 bp downstream | |
| >P4 | | 127 | No similarity sequence | Dpox3 DNA | 776 bp downstream | |
| >P5 | 5, 14 | 301 | <i>Elaeis guineensis</i> actin depolymerizing factor | | | |
| >P6 | 8 | 160 | Peroxidase 47 | | | Phenylpropanoid biosynthesis pathway |
| >P14 | 16 | 148 | Glutamine synthetase | | | Nitrogen metabolism biosynthesis |
| >P15 | 10 | 136 | WRKY transcription factor | | | |
| >P29 | 1 | 660 | No similarity sequence | Uncharacterized protein | 482 bp up-stream | |

LG: Linkage group based on oil palm genome published by Singh et al., 2013, bp: Base pair

Table 2: Recapitulation of sequence analysis using Blas2GO software

| Gene names | Biological process | Molecular function | Cellular component |
|--|---|--|---|
| <i>Elaeis guineensis</i> actin depolymerizing factor | Responses to oxidative stress, actin filament depolymerization, cell growth, responds to salt stress, cell morphogenesis, glycolysis, gluconeogenesis, responds to cadmium ion | Actin binding | Cytosol, mitochondria, plasmodesma, actin filament, chloroplast, nucleus, plasma membrane |
| Peroxidase 47 | Oxidation-reduction process, responds to oxidative stress | Heme binding, peroxidase activities | |
| Glutamine synthetase | Nitrogen fixation, Glutamine biosynthesis process | Ammonia-glutamate ligation activities, ATP binding | Cytoplasm |
| WRKY transcription factor | Cellular response to phosphate starvation, responds to Gibberellin stimuli, jasmonic acid-mediated signal, responds to hydrogen peroxide, Nitric oxide-mediated signal transduction | Ion Zn binding, calmodulin binding, | Vacuola membrane, apoplast, nucleus, plasmodesma |

peroxidase is an intracellular and class III is generally secreted to cell wall and vacuole (Welinder et al., 2002). Plant class III peroxidase constitute of large number of gene family (Passardi et al., 2004) and each isoform might play a specific function (Cosio and Dunand 2008). One of the POD function in plant is known to be involved in lignification process in tobacco (Marjamaa et al., 2008).

Lignin biosynthesis is a branch of phenylpropanoid biosynthesis (Boerjan et al., 2003). Lignin polymer consist of 3 p-hydroxycinnamyl alcohol are p-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol which mediated by enzyme laccase and class III POD (Barcelo 1997). Phenol or polyphenol was synthesized through shikimate pathway (Knaggs, 2001). Phenolic compound in plant consist of benzoquinone, phenolic acid, flavonoid, coumarins and lignin (Douglas 1996). These compounds are important in growth, production, resistance to biotic stress, tolerance to a-biotic stress such as drought and temperature stress (Aoki et al., 2000).

Some enzymes activities namely SOD, Catalase, Ascorbate peroxidase and peroxidase are increased during drought stress in both leaf and root of olive trees (Sofa et al., 2005). Lignification in plants may occur in response to stress. Activities of enzymes *guaiacol peroxidase*, *coniferyl alcohol peroxidase* and *syringaldazine peroxidase* are also increased during drought stress (Lee et al., 2007).

Enzyme peroxidase fragment in cDNA hard bunch fruit may involved in lignin biosynthesis in fruit abscission zone. Lignin concentration in abscission zone of hard bunch fruits is higher and significantly different compared to fruits of normal bunch (data being published). While that of in the other zones (pedicle and mesocarp) the lignin concentration was not significantly different. The differences of lignin concentration in closely located in the fruit are common. Lignin decomposition process can be different between taxa, species, and cell type (Nakashima et al., 2008), may even differ between cell wall layers (Gou et al., 2008).

WRKY transcription factor was involved as an important signaling regulation in plant metabolism, such as response to biotic, abiotic, senescence and seed development (Rushton et al., 2010). WRKY play important roles as a response to abiotic stress such as freezing, heat, drought and salt stress. Activation of WRKY transcription factor in hard bunch caused by exceeding temperature and length of sunshine in Lampung (Roberdi 2014). The involvement of WRKY transcription factor in improving tolerance to drought in *Arabidopsis* occurred through the increased of ABA concentration (Jiang et al., 2012). Abiotic stress is regulated by ABA and it is known that WRKY is a key factor in signaling pathway which responsive to ABA (Rushton et al., 2011). WRKY together with other TFs such as MYB, bHLH, bZIP, ERF, NAC interact with each other in response to drought stress (Tripathi et al., 2014).

Another TDF obtained in this study was an *actin depolymerising factor* (ADF), an actin binding protein which has small size in molecular weight ranged between 15-22 kDa (Lappalainen et al., 1998) that regulates actin dynamics in cells. In *Arabidopsis* ADF has 11 isoforms protein functional and categorizing into four sub-classes based on tissue specificity and expression (Ruzicka et al., 2007). Rice and *Arabidopsis* ADF have high similarity based on action, physiology function and expression pattern (Feng et al., 2006). ADF with other *actin binding protein* modulate activity of actin filament intracellular. Actin and ADF work in the cytoplasm and nucleus, which controlled various cellular function and development of organism. Based on proteomic analysis, ADF was reported to be involved in tolerance mechanism and as a protein induced by drought stress in rice (Salekdeh et al., 2002). In other report, expression of actin genes are increasing during ripening of oil palm fruit (Teh et al., 2014).

Glutamine synthetase (GS) is widely distributed in the plant. This enzyme exist in two main forms, such as chloroplast GS and cytosolic GS. Main function of GS is involved in nitrogen metabolism in plants. While cytosolic GS assimilates ammonium derived from Nitrogen uptake

and Nitrogen recycling pathway (Bernard and Habash, 2009). GS is a key enzyme involved in proline synthesis. Proline is an osmoregulator which accumulated when plant exposed to drought stress (Sankar et al., 2007). GS is a good indicator for wheat tolerance to drought and can be used as a indicator in characterization of wheat tolerance to drought (Nagy et al., 2013).

CONCLUSION

cDNA-AFLP method has been proven to be a convenient and effective technique for the transcriptomic comparison of different genotypes, without prior knowledge of the genome sequence. This analysis has identified some putative target genes which are thought to be involved in the adaptation to water stress that caused hard bunch phenomena in Southern of Sumatera. Four of nine sequences had similarity with sequences namely *actin depolymerizing factor*, *peroxidase-like*, WRKY transcription factor and *glutamine synthetase*. All the TDFs identified here are involved in response to drought stress, which supports our hypothesis that the hard bunch phenomenon could be caused by water deficiency.

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Authors contributions

R. designed and conducted most of the experiments, analyzing the results and preparing the manuscript. S. Y. and N. T. M. were involved in editing the manuscript and supervising R. doctoral research activities. S was involved in data analysis and presentation, manuscript preparation and editing. He is the major (chairman) of the supervisory committees of R. doctoral program.

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