Physiology and genotyping of adaptive and sensitive oil palm progenies under unwatered stress condition

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Abstract. Increasing of dry areas for Oil Palm plantation due to climate change led the breeders to develop drought stress tolerant palm. Characterization of genetic materials for their physiological and genetic properties is required. Two distinct progenies derived from Tanzanian origin used for more adaptive to unwatered stress, and Nigerian origin used for more sensitive, were subjected for analysis. Physiological related variables were observed for sap flow, water potential, soil water content, greenhouse temperature and relative humidity, stomatal density, stomata pore width, and proline content. The Tanzanian origin has more stable in sap flow, longer water potential detected, more adaptive in stomata closure, and a lower in proline content than Nigerian origins. The genome comparison of both materials was performed by double digest Restriction Associated DNA and captured 299 294 SNPs. It found 9.3 % of those SNPs that were having a different allele with a minor frequency between progenies. And, 0.7 % from filtered SNPs were located and distributed in 167 genes. Two SNP markers were located in aquaporin NIP1-1 gene that related to water translocation between cells. One SNP marker was located in glutamate receptor 2.7-like gene that related to glutamate release and proline biosynthesis. The remaining SNPs will be in further analyzed.

Keywords: Aquaporin, glutamate, proline, sap flow, SNP, stomata, water potential

1. Introduction

Oil palm (OP) is more suitable grown in areas with 2 000 mm evenly spread annual rainfall [1]. The OP inflorescence development takes about 2 yr [2]. The dominance of male inflorescence formation and female flower abortion will occur when it is insufficient rainfall [3]. Some OP plantations in
Indonesia have a low amount of rainfall in September 2018 (figure 1) [4]. Two major negative impacts occur at different phases of inflorescence development in the adult OP. First impact, drought stress condition at the palm sex determination phase will induce dominance of male inflorescence and decrease fresh fruit bunch (FFB) harvesting about 28 mo after stress. Second impact, drought stress condition at the female flower development phase will induce female flower abortion and decrease the FFB harvesting about 12 mo after stress [3].

The OP production could decline due to the water deficit from the low rainfall [5]. The major impact of drought stress on OP production stimulates the breeding and selection OP which is more tolerant to drought stress. The breeding method that used for selection is multi location progenies test and observe some phenotypic that related to drought stress [6]. Another method is to use some molecular markers that assisted the selection [7]. This last method needs appropriate markers to do the selection.

Selection of genetic markers to distinguish the drought tolerant palms could be performed by comparing the genome profile of distinct genetic material. The physiological responses under unwatered and watered condition are incorporated to support the data. Subsequently, two distinct progenies in tolerance to unwatered stress were compared to screen the single nucleotide polymorphism (SNP). Some phenotypic variables and genome between progenies were compared in this research.

2. Methods

2.1. Progeny selection and general observed variables
Two distinct progenies under unwatered stress condition were used in this study. The progeny with Tanzanian genetic background was selected as a more adaptive progeny to the stress while the progeny with Nigerian genetic background was used as a more sensitive progeny to the stress. Both progenies were selected according to the preliminary study involving five different progenies (table 1). The progenies were assigned for watered and un-watered treatment for 1 mo with three biological replicates in the greenhouse (figure 2).

One year old seedlings with contrast genetic background were subjected for watering treatment in the greenhouse. All palms were fully-irrigated until reach the field capacity condition before treatment and set the day as Day-0. Watered treatment was performed with a supply of 800 mL of water every day. Unwatered treatment was performed without giving water to palm media.
Table 1. Five progenies that treat under unwatered stress preliminary study

| Progeny code | Mating design | Genetic composition |
|--------------|---------------|---------------------|
| 003          | TAN 622       | Tanzania 25% - Compacta 25% - Avros 50% |
| 007          | NIG 376       | Deli 25% - Nigeria 25% - Avros 50% |
| 008          | COM 650       | Deli 25% - Compacta 25% - Avros 50% |
| 011          | COM 696       | Compacta 25% - Nigeria 25% - Deli 25% - Ekona 25% |
| 016          | GHA 536       | Deli 50% - Ghana 50% |

Figure 2. Five progenies test in green house; unwatered stress condition and the 003 progeny was having more green leaf than others (left) and the control (right).

Physiological responses were estimated by measuring plant sap flow, plant cell water potential, the stomatal density, the average of stomatal pore width, and the proline content. The soil water content of plant growth medium, green house temperature and relative humidity were recorded. Each palm from both progenies was genotyped using the Double Digest Restriction Associated DNA (ddRAD) method to observe the allelic variation.

2.2. Physiological variables observation

Several physiological response variables were observed using the tools provided by ICT International. Palm sap flow was estimated at the petiole in the third frond of all palms using the Sap Flow Meter (SFM). Cell water potential was estimated at the petiole in the second frond of two palms using the Psychrometer (PM); those palms consist of one palm from each progeny under unwatered treatment. The soil water content of palm media was observed with Soil Moisture Meter (SMM) which was installed at 15 cm from polybag base in all palms (figure 3). Air temperature and relative humidity in the greenhouse were recorded with Automatic Weather Station (AWS). All tools were adjusted to record the data within 30 min of interval.

2.3. Stomata

The stomatal measurement was performed at the Day-21st. Leaf samples were taken from the 5th and 6th leaf at first frond. Sampling time was performed in four consecutive times daily, at 9:00, 12:00, 14:00, and 19:30 o’clock. Fresh leaves samples were immediately stored in FAA 70% solution (5% of 4% formaldehyde + 70% of 95% alcohol + 5% of 100% glacial acetic acid + 20% of aquades). Abaxial surface of leaf samples was observed using a light microscope. Stomatal density and width of stomatal pore were measured; stomatal density was observed under 400 times magnification while stomatal pore was observed under 1 000 times magnification.

2.4. Proline content
Proline content was estimated from leaves at the end of the second frond. Samples were measured three times, i.e. Day-0 morning, Day-20 morning, and Day-20 afternoon. Leaf samples were stored immediately in cold box prior to pulverization. Leaf powder of a half gram was put into a reaction tube with 10 mL of 10 % sulfosalicylic acid solution and being agitated for 1 min in a vortex machine. Whatman 41 filter paper was used to separate debris and the supernatant. Subsequently, 2 mL of supernatant was reacted with 2 mL Ninhydrin acid and 2 mL of glacial acetic acid, and incubated for 1 h at 100 °C, afterward the reaction was terminated in an ice bath. The solution was extracted with 4 mL of toluene and being agitated for 20 s in a vortex machine to forming a chromophore at the solution surface. The absorbance of chromophore was measured with a spectrophotometer at 520 nm wave length. Proline content was calculated in mg g$^{-1}$ of leaf weight, by analyzing the absorbance of chromophore from samples with spectrophotometer [8].

![Figure 3. The 003 and 007 progenies under unwatered stress and were instaled with ICT tools. PM = Psycrometer, SFM = Sap Flow Meter, and SMM = Soil Moisture Meter](image)

2.5. The ddRAD genotyping and SNP mining

The collected young leaves from the second frond were extracted for genomic DNA analysis using the NucleoSpin® Plant II mini kit (Macherey-Nagel, Germany) in accordance with the manufacturer’s protocols. The purity and quantity of DNA were estimated by the NanoDrop® 2000 UV-Vis Spectrophotometers (Thermo Scientific, USA). DNA quality was checked by using the 1 % agarose based electrophoresis.

The genomic DNA was digested with both EcoRI (recognition site 5’-G/AATTC-3’) and MseI (recognition site 5’-T/TAA-3’) restriction enzymes. After digestion, the ends of DNA fragments were ligated with barcoded adapters using the DNA ligase. The ligated DNA was pooled, and after subsequent purification, the libraries were then enriched using PCR to increase the fragment pools. The PCR products were cleaned up and checked for fragment size on a DNA analyzer. Further, the RAD-seq libraries were sequenced using an Illumina hiseq platform for single-end (1 × 150 base pair).

Clean sequence reads from FASTQ files were mapped into EG5 NCBI reference genome [9] using the BWA-MEM [10] algorithm under the Burrows-Wheeler Aligner (BWA) software with a default parameter. Furthermore, under the SAM Tools package, the alignment file was sorted and indexed. Mpileup was used as SNP caller. Vcftools was used for filtering the SNPs. In order to reduce the low-quality genotypes, only the SNPs with at least 16x sequence depth and lower than 20 % missing data were used for further analysis. Furthermore, the remaining SNPs were re-filtered with the minimum of Minor Allele Frequency (MAF) and remove the non bi-allelic loci. Whole bi-allelic SNP variances that generated from the ddRAD genotyping were filtered for allele composition within the Tanzanian as well as Nigerian origin progenies.

2.6. In silico analysis of selected SNPs for RNA structure and protein function prediction

Prediction of RNA structural changes based on SNP variation was performed using RNAsnp software [11]. Three modes were used for calculating the effect of SNP on the RNA structure. The first mode was designed to predict the effect of SNPs on short RNA sequences while the second mode was designed to predict the effect of SNPs on large RNA sequence. And, the third mode, as the
combination of first and second mode, was designed to screen all possible structure-disruptive SNPs in an input sequence using a brute-force approach.

Prediction of protein function change by sequence variation was performed using PROVEAN software [12]. Clustering of BLAST hits was performed by CD-HIT with minimum 75% global sequence identity. The top 30 clusters of closely related sequences form the supporting sequence set, which was used to generate the prediction. A delta alignment score was computed for each supporting sequence. The scores were then averaged within and across clusters to generate the final PROVEAN score. If the PROVEAN score was equal to or below a predefined threshold, the protein variant was predicted to have a "deleterious" effect. If the PROVEAN score was above the threshold, the variant was predicted to have a "neutral" effect.

3. Result and discussion

3.1. Greenhouse temperature and relative humidity

Greenhouse air temperature (T) and relative humidity (RH) during the unwatered experiment were ranged from 21.8 °C to 39.3 °C and 39.7 % to 94.4 % (figure 4.A). There was a negative correlation between T and RH (r = -0.9**). During the night time, T average was decreased to 25.6 °C and RH average was increased to 79.6 %. In the afternoon, T average was increased to 32.8 °C and RH average was decreased to 56.8 %. The increasing of T was correlated to the significant increasing of sap flow in the watered palm samples (r = 0.7**). T and RH parameters were known to influence plant evapotranspiration and had a significant effect on growth and physiological cycles [13].

3.2. Sap flow, water potential, and soil water content of watered samples

In the watered samples, frond sap flow has positive flow in all time, about 4.5 cm h-1 in the night and 29 cm h-1 in the afternoon. The frond cell water potential was around -0.1 MPa in the night and gradually decreased in the afternoon to -1.5 MPa. The decreasing of cell water potential was related to osmolite cell production. The solutions which participated in osmotic adjustment were inorganic ions (mainly K+ and Cl-) or uncharged organic compounds like proline or glycine betaine, as well as carbohydrates like sucrose, pinitol or mannitol [14]. It seems that the water potential was correlated to sap flow with r = -0.8** (figure 4.B; C; D).

The soil water content of palm media was maintained within 30 % to 45%. The decreasing soil water content was ranging from 4 % at night to 10 % in the afternoon. The higher decreasing soil water content in the afternoon was correlated to T, RH, and sap flow (r = -0.6**) in the palm. There was no significant different for sap flow and water potential between progenies under watered treatment.

3.3. Physiology variables of unwatered samples

In the unwatered samples, there was a different sap flow rate between both progenies. The frond sap flow of ex-Tanzania progeny had positive flow in all time, about 4.4 cm h-1 in the night and 5 cm h-1 in the afternoon. Interestingly, the frond sap flow of ex-Nigerian progeny was negatively detected in the late 16 d to 20 d by -5 cm h-1 at night (figure 4.C). The negative sap flow could be occured by over production of osmolite and remobilized resources to new root or frond [15]. Higher proline content was occured in both progenies when treated with unwatered stress (figure 5). This was similar with 18 mo old OP seedlings under drought stress that have significant proline content increment compared to the control [16]. However, high proline content detected in the ex-Nigerian leaves samples at Day-20, about 33.6 fold from watered palm samples, and 2.5 times higher than ex-Tanzanian progeny. This might explain the negative sap flow in the night of the ex-Nigerian progeny.
The frond cell water potential of ex-Tanzanian progeny was detected until the end of treatment in Day-27, around -5 MPa in the night and -8.2 MPa in the afternoon. The frond cell water potential of ex-Nigerian progeny only detected until about Day-20 and was predicted that occurs earlier cell death than ex-Tanzanian progeny around the sensor. The significant decreasing of cell water potential was related to a more limited amount of water that can be absorbed by palms and adaptation of stomata under unwatered stress condition. The soil water content of unwatered media was gradually decreased from 34.4 % at Day-0 to 19 % at Day-27. Stomatal closure is one of palm adaptations under stress. There was different stomatal closure ability between progenies (figure 6) while no significant different in stomatal density. In the afternoon of Day-21, the stomatal organ of ex-Tanzanian progeny was more adaptive to keep the water by hold the pore about 1.4 μm than 007 progeny about 4.8 μm (figure 7). Based on this phenomenon, it was assumed that more water loss of ex-Nigerian progeny than ex-Tanzanian, and became one reason that higher proline content and earlier cell death that was occured in the ex-Nigerian progeny.

Figure 4. Green house air temperature and relative humidity (A); sap flow rate of ex-Tanzanian (003) progeny (B); sap flow rate of ex-Nigerian (007) progeny (C); cell water potential between both progenies (D) during 27 d treatment.
Figure 5. Leaf proline content of ex-Tanzanian (003) and ex-Nigerian (007) progenies (left), and proline content fold change between unwatered and watered treatment (right).

Figure 6. Stomata cell observation of leaves samples that take in the afternoon; adaptive progeny has a smaller stomatal opening (top right image) compared to the sensitive progeny (bottom right image).

Figure 7. The dynamics of the stomatal opening of ex-Tanzanian (003) and ex-Nigerian (007) progenies.
3.4. Genome comparison for SNP selection

Whole 299 294 SNPs were captured by comparing the ex-Tanzanian and ex-Nigerian genotyping results (table 2). Furthermore, 27 962 SNPs (9.3 %) were passed to the filtering on minor allele frequency (MAF) between both progenies. Out of selected SNPs, only 202 SNP markers (0.7 %) were located in the annotated genes.

Table 2. List of SNPs mined from ex-Tanzanian and ex-Nigerian genome comparison

| Pseudo Chromosome | SNP candidates | Selected SNP by MAF | SNP located in the annotated gene |
|-------------------|----------------|---------------------|----------------------------------|
| 1                 | 10 934         | 1 364               | 34                               |
| 2                 | 11 400         | 1 153               | 25                               |
| 3                 | 9 858          | 1 061               | 22                               |
| 4                 | 9 638          | 1 046               | 18                               |
| 5                 | 8 920          | 1 104               | 13                               |
| 6                 | 7 233          | 846                 | 11                               |
| 7                 | 7 074          | 805                 | 17                               |
| 8                 | 6 693          | 708                 | 11                               |
| 9                 | 6 087          | 714                 | 6                                |
| 10                | 5 602          | 694                 | 8                                |
| 11                | 4 462          | 631                 | 5                                |
| 12                | 5 491          | 574                 | 8                                |
| 13                | 4 877          | 579                 | 4                                |
| 14                | 4 222          | 481                 | 2                                |
| 15                | 4 219          | 468                 | 2                                |
| 16                | 3 971          | 488                 | 2                                |
| Chloroplast       | 140            | 5                   |                                  |
| Unscaffold        | 188 473        | 15 241              | 14                               |
| **Total**         | **299 294**    | **27 962**          | **202**                          |
|                   |                | **9.3 %**           | **0.7 %**                        |

Three SNPs were located in the unwatered tolerance related genes whereby one SNP was located in the glutamate receptor gene in the pseudo-chromosome 2, and two SNPs were located in the aquaporine gene in the pseudo-chromosome 3 (figure 8). Glutamate receptor was involved in the glutamate release mechanism in the cell [17]. The SNP was located at the second exon of the glutamate receptor 2.7-like gene, and the translation of the SNP mutation will change the predicted amino acid from Arginine to the Glutamine (table 3). Simulation for RNA structure using the RNAsnp software has shown a non-significant effect of SNP. Further in silico simulation for protein function using PROVEAN software has shown a neutral effect of SNP. For plant adaptation to the abiotic stresses, the glutamate production was essential as a precursor for proline synthesis [18, 19]. Proline biosynthesis through glutamate dependent pathway occurred in the cytosol and mediated by P5CS and P5CR enzymes [20].
Table 3. Selected SNP for further verification.

| ID     | CHR | Pos (cM) | Annotated Gene                  | MAF   | SNP | Type       | Amino Acid change | RNAsnp Score | PROVEAN Score | Remark  |
|--------|-----|----------|---------------------------------|-------|-----|------------|-------------------|---------------|--------------|---------|
| SNP.25853 | 2   | 52.0     | Glutamate receptor 2.7         | 0.38  | G/A | missense   | R to Q           | 0.297        | ns           | -1.232  |
| SNP.30143 | 3   | 8.2      | Aquaporin NIP1-1              | 0.25  | A/G | missense   | E to G           | 0.095        | Significant | -0.696  |
| SNP.30145 | 3   | 8.2      | Aquaporin NIP1-1              | 0.25  | T/G | missense   | F to C           | 0.041        | Significant | 0.626   |

Two SNPs were located at the first exon of NIP1-1 Aquaporin gene in the pseudo-chromosome 3 (figure 8). Both SNPs were haplotype. Therefore the selection of SNPs for further molecular marker could be selected either one. The translation of both SNP mutation will change the predicted amino acid (table 3). Simulation for RNA structure using the RNAsnp software has shown a significant effect of SNP ($P = 0.095$ and $0.041$). In the silico simulation for protein function using PROVEAN software has shown a neutral effect of SNP. The Aquaporin plays a significant role in the adjustment of stomata cell guards in the drought stress condition [21]. In rice, aquaporin has a contribution to ABA-triggered stomatal closure through OST1-dependent phosphorylation [22]. In Arabidopsis thaliana (L.) Heynh., ABA-and pathogen-triggered stomatal closure was influenced by aquaporins [23]. In Grapevine, NIP1-1 aquaporine gene can regulate water transport across roots as such that transpirational demand is matched by root water transport capacity [24].

Figure 8. The number of SNPs within 1Mb window size and three SNP markers selected

4. Conclusion
All physiological parameters response of adapted (ex-Tanzanian progeny) and sensitive (ex-Nigerian progeny) genetic materials showed a different mechanism to the unwatered stress condition. Ex-Tanzanian progeny has consistently in sap flow rate, longer water potential detection, adaptive of stomatal closure, and lower proline production than ex-Nigerian progeny. Whole 202 SNPs were selected by comparing the genome profile between both progenies. The SNPs were distributed over 167 genes whereby two genes seem to have a relationship with the physiological parameters recorded,
namely aquaporin and glutamate receptor genes. Two SNP markers were located in Aquaporin NIP1-1 gene with significant effect on the RNA structure, while one SNP in Glutamate receptor 2.7-like gene seems to have a non significant effect to the RNA structure.

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