Phenotypic and molecular changes in cassava (*Manihot esculenta* Crantz.) genotypes as a response to water-deficit stress

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**Abstract.** Three local Indonesian cassava (*Manihot esculenta* Crantz) genotypes, Adira 4, Roti, and Ubi Kuning retained their growth under a water deficit environment. To understand the physiological and molecular responses of cassava plants under such stress, we observed leaf stomata characteristics, i.e. stomata dimension (width and length) and density, and molecular expression of *aquaporin* (*AQP*) gene, respectively. The observation was performed on all genotypes on day 26 and 45 after water-deficit treatment. Quantification of gene expression was performed by comparing the threshold cycle (Ct) values of *AQP* with Ct values *β-tubulin* for each genotype. Results showed that water deficit treatment significantly affected both stomata density, length and width in all genotypes. On day 26 after treatment, the density of stomata in Roti and Ubi Kuning increased two times higher than the stomata density in plants under well-watered supply. Stomata length of Roti and Adira 4 under water stress was 0.4 times shorter than those under normal water supply. Relative expression of *AQP*, encoding *AQP PIP2* proteins, in the Roti genotype showed that the gene transcripts have no significant elevation after experiencing water stress. In Adira 4, the expressions of *AQP* increased two-times at day 26 under water-deficit treatment compared with *AQP* expressions in well-watered treatment and continued to elevate up to three times higher at day 45 than those of expressed in Adira 4 plants under well-water treatment. In addition to stomata phenotypic changes, Ubi Kuning may regulate its water channel proteins *AQP PIP2* to control turgor pressure in the beginning phase of water stress conditions. These results showed that three cassava genotypes responded differently under water deficit stress and these may serve as basic knowledge for further analysis.

1. Introduction

Issues on global warming and climate changes have become a great concern in the world because the unpredictable seasons and uncertain rainfall lead to several abiotic stress, i.e. drought (water deficit), flood (water excessive), salinity and extreme temperature. Drought can limit the availability of water resources and arable land. This could diminish plant growth and its yields are up to 70% worldwide [1]. Plants have evolved sophisticated mechanisms to respond to abiotic stresses, including morphological, physiological and biochemical changes during their growth and development process [2]. Biochemical and physical changes often found in plants as responses to drought, including abscisic acid (ABA) accumulation, stomatal closure, water potential alteration, photosynthetic rate
decrease and plant solute accumulation [3, 4, 5]. Stomata play a major role in gasses exchange and transpiration, as well as control water and carbon dioxide exchanges between the plant and the environment [6]. Some plants mitigate the water-deficit environment by modulating the pore size of stomata and regulating numbers and positions of stomata as well as the capacity to open and close; in order to maximize the photosynthetic efficiency as well as to minimize the water loss [6, 7, 8]. Alteration in stomata density and dimensions (width and length) due to drought stress has been recorded in wheat and rice [6, 9].

Responses to drought stress in plants are regulated by genes encoding molecules or proteins, such as transducer signal of kinases and phosphatases, transcription factors (TF), and intrinsic membrane proteins, such as aquaporins [10]. Aquaporins are water channel proteins in cell membranes that facilitated passive transcellular transport of water or small neutral molecules [2, 11]. Aquaporin proteins could be grouped into plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs) [11, 21]. PIPs expressions have been reported to play a role in reducing leaf transpiration, including Phaseolus vulgaris PIP2 [11]. In addition to water channel protein function, Vitis vinifera PIPs, i.e. VvPIPI and VvPIP2, are also important in increasing water permeability three times higher than that in normal conditions [12]. In cassava, 17 transcripts homolog to aquaporin Arabidopsis increases after drought-stress treatment, in addition to TIP2 homolog [10]. However, the transcripts have not been identified further and it is important to identify genes underlying drought tolerance mechanisms in cassava.

Cassava is one of the important crops producing starch after rice and maize. Cassava starch is used in a wide range of industrial applications, including food, paper, textile, plywood, glue and alcohol [23]. Cultivation of this crop can be done under marginal conditions, including low fertility soils and areas that face sporadic or seasonal drought [13]. Cassava can also endure several months of natural drought during its seasonal cycle and tolerant to long periods of water shortage, which are still able to produce high yield on dry land compared with other food crops such as rice and maize [3]. In general, cassava can be classified as a drought-tolerant plant. Morphological changes due to water deficiency were observed starting day 45 after water limitation treatment in cassava plants, such as the decrease of leaf numbers, a halt in stem growth, the decrease of mature leaf areas, and the number of leaves stayed green color [3, 30]. Plant response to water deficiency varies between species, genotypes or cultivars. For example, cassava cultivar Huay Bong 60 retained the numbers of stomata to be open than cultivar Hanatee under the same water-deficit treatment [31].

This study aimed to profile responses of three Indonesian cassava genotypes (Adira 4, Ubi Kuning, and Roti) under water-deficit treatment, i.e., stomata characteristics and aquaporin gene expression. Plant responses were observed at day 26 and 45 after water-deficit treatment. The observation periods used in this study were based on the previous study showing the significant plant responses to water-deficit treatment in cassava genotypes included in this study were observed at day 45 after the treatment [30]. Our results showed a variation of stomata characteristics and aquaporin expression in three cassava genotypes. The obtained knowledge will provide information for further improvement of cassava cultivar with better adaptation to drought conditions.

2. Material and Methods
2.1. Plant material and growth condition.
Three cassava genotypes, i.e. Adira 4, Roti, and Ubi Kuning were cultivated in individual plastic pots (15,000 cm³), containing soil mixed with compost (2:1; v/v). The plants were grown in a greenhouse located at Research Center for Biotechnology LIPI, Cibinong. The experiment was designed in a factorial experiment with two factors, i.e., water-deficit treatment and well-watered treatment, each contained seven replicates per genotypes. All plants were watered three times a week with 1200 cm³ for three months. Water deficit treatment was performed using a protocol described in Catalayud et al. 2000 [3] by decreasing the irrigation volume from 1200 (control) to 80 cm³ (drought treatment) for 45 days. Well-watered treatment was performed by irrigating the plants three times a week with 1200
The environmental condition was maintained approximately 30/25°C (day/night) with natural periods (between 13 to 16 hours) and 70-80% relative humidity. Plants were measured their stomata characteristics and isolated their total RNAs at 26- and 45-days during water stress treatment following the experiment of Calatayud et al. 2003 and Hartati et al. 2013 [3, 30].

2.2. Stomata density (mm²) and Size (µM)
Third leaves after the apical shoot were collected from all plants on days 26 and 45 at 9.00 am during the treatment. Stomata characteristics, i.e., density, length, and width, were observed from the abaxial surface of leaves using an inverted microscope (LEICA Microsystem). Before observation, the abaxial surface was coated with nail varnish (formaldehyde and toluene-free) and let the surface until dry. The coated leaf was peeled off, mounted in the water on a glass slide, and covered by a cover glass. Stomata density was observed by counting stomata present in three randomly chosen microscopic fields for each slide. One microscopic field consisted of 0.32066 x 0.23957 mm² at 40x magnification. The number of stomata per field was converted to the number of stomata per mm² and this value described stomata density. Stomata length and width were observed by measuring the aperture length and the distance of two guard cells next to aperture, respectively, using an ocular calibrated micrometer in 10 randomly chosen microscopic fields from each slide at the magnification of 400x on abaxial leaf surfaces (Figure 1). Data were then analyzed by a mixed-model ANOVA and mean comparison by the Duncan’s test using SPSS software (SPSS for windows 16.0 version).

2.3. Total RNA Extraction, cDNA Synthesis, and quantitative real-time PCR analysis
The 50 mg cassava leaves collected at day 26 and 45 after treatments were subjected to RNA extraction for further analysis. The RNA extraction was done using Plant Total RNA Mini Kit (RP100; GeneAid). Total RNA was measured using the NanoPhotometer™ P-Class P 300 followed by running the RNA in gel electrophoresis with 1.5 % gel agarose with 1xTAE buffer. The cDNA synthesis was done by subjecting 0.5 µg of total RNA to the reverse transcriptase (RT) reaction using Taqman® Reverse Transcription (Applied Biosystem). Ten nanograms of cDNA from the reaction were then used for quantitative real-time PCR analysis (qRT-PCR). Three genes were targeted for qRT-PCR analysis, which was the Aquaporin PIP2 gene (EU599222.1; Manes.04G076500.1), β-tubulin (BTUB; Manes.05G147000.1) and Elongation Factor1-alpha (EF1-A; AF041463.1). Oligonucleotides for each gene were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer_3plus.cgi) by following the recommendation from Udvardi et al. 2008 [22]. Oligonucleotide sequences were described in Table 1. The qRT-PCR analysis was done using Eco™ Real-Time PCR System (Illumina) machine. Real-time PCR run time was 40 cycles consisted of pre-denaturation at 95°C for 5 min, denaturation 95°C for 3 sec, and annealing 60°C for 30 sec. The qRT-PCR analysis was done using SYBR® FAST qPCR Kit Master Mix (2x) Universal (KAPA Biosystem; KK4601). The composition and volumes of the qPCR reactions were according to the manufacturer’s instruction. qPCR analysis was performed with three biological replicates of each
acccession. Expression levels were determined by delta Ct (ΔCt) values, calculated by subtracting Ct value of each candidate gene with Ct value of BTUB, converted by 2ΔCt calculation, multiplied by 100. Relative gene expression values were presented in log2 scale. Statistical analysis was conducted by comparing the mean of each biological replicates (n = 3) of each accession using One-way ANOVA and continued with Duncan’s test (a = 0.05). This was performed in IBM® SPSS® Statistics version 25.

Table 1. Gene annotation and primer sequences of aquaporin PIP2, β-tubulin, and Elongation Factor1-alpha for gene expression analysis

| Genes                           | Gene ID code in NCBI/ Phytozome v12.1 | Oligonucleotide sequence 5’ to 3’ |
|---------------------------------|---------------------------------------|----------------------------------|
| Aquaporin PIP2                  | EU599222.1/ Manes.04G076500.1          | forward: TTTCACTGGGAGCACTTGCAG   |
|                                 |                                       | reverse: AAGATCCCAAAGCTTGATG      |
| β-tubulin (BTUB)                | Manes.05G147000.1                      | forward: GCAACATGAACGATTGGTG     |
|                                 |                                       | reverse: GCCCTCGTCTCATACTCAA      |
| Elongation Factor1-alpha        | AF041463.1                            | forward: CTTTCTCTGCGTACCCCTCAA   |
| (EF1-A)                         |                                       | reverse: TGATCACACCAACAGCAACC    |

*Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html#)

3. Results and Discussion

3.1. The stomata characteristics of cassava genotypes under drought treatments observed at 26 and 45 days

Stomata characteristics of Roti, Adira 4, and Ubi Kuning at day 26 and 45 after water-deficit and well-watered treatments were recorded in tables 2 and Figure 3. The observation intervals, day 26 and 45 after water-deficit treatment, were based on results presented in Calatayud et al. 2000 and Hartati et al. 2013 [3, 30]. The studies described cassava plants showed severe levels of morphological and physiological changes at day 45 after water-deficit treatment [3, 30]. In addition, Hartati et al. 2013 showed that cassava plants were losses of their leaves started at 4 weeks after water-deficit treatment. In this study, we observed similar responses to those observed in Hartati et al. 2013. We observed the severity of plant damage at day 45 after water-deficit treatment (Figure 2).

Observation of stomata density was performed at the abaxial leaf surface. This was due to the abaxial leaf surface contains more stomata than that on adaxial leaf surface and gas exchange occurs highly on abaxial stomata, suggesting that abaxial stomata were more sensitive to environmental perturbation [24, 25]. In general, stomata densities in all genotypes were similar under well-watered condition. Stomata densities in Roti and Ubi Kuning increased two-times higher under water-deficit treatment than those under the well-water condition on day 26 (table 2). Increasing in stomata densities after water stress treatment also shown as those in a drought-stress-legume plant, *Vicia alba* [18]. More densities in stomata occurred in *V. alba* were apparently as one of the mechanisms to regulate the atmospheric gas exchange, thus, they may reduce water usage under drought stress and facilitate the water uptake under water stress [18]. However, other plants performed different mechanisms under water stress conditions, as shown in rice [17]. At day 45 under water-deficit condition, all genotypes showed similar stomatal densities, indicating that the plants had performed optimum response mechanisms under the stress condition. In contrast, stomata densities of Adira 4 were the same in both water conditions, suggesting that water deficit-treatment did not influence
amounts of stomata in this genotype. In several plants, stomata densities appear to correlate with genotypic and environmental variation, including the atmospheric CO₂ concentration [14, 15, 16].

![Images of Adira 4 (A), Ubi Kuning (B), and Roti (C) under normal water condition (left) and water-deficit treatment (right) at day 26](images)

**Figure 2.** Plant profile of Adira 4 (A), Ubi Kuning (B), and Roti (C) under normal water condition (left) and water-deficit treatment (right) at day 26

**Table 2.** The differences of stomata characteristics among three genotypes of high beta-carotene cassava observed at 27 and 45 days under drought stress.

| Days of observation | Treatments       | Genotypes   | Stomata density (mm²) | Stomata dimensions |
|---------------------|------------------|-------------|-----------------------|--------------------|
|                     |                  |             |                       | Length (µm)        |
|                     |                  |             |                       | Width (µm)         |
| 26                  | Well-watered     | Roti        | 373.33 ± 37.36        | 19.12 ± 0.76       |
|                     | treatment (mm²)  | Adira-4     | 364.67 ± 34.71        | 15.19 ± 0.62       |
|                     |                  | Ubi Kuning  | 268.67 ± 26.36        | 13.40 ± 0.74       |
|                     | Water-deficit    | Roti        | 616.33 ± 22.93        | 11.09 ± 0.58       |
|                     | treatment (µm)   | Adira-4     | 377.33 ± 39.33        | 13.61 ± 0.36       |
|                     |                  | Ubi Kuning  | 586.00 ± 75.06        | 13.55 ± 0.35       |
| 45                  | Well-watered     | Roti        | 359.67 ± 15.62        | 16.76 ± 0.91       |
|                     | treatment (µm)   | Adira-4     | 580.67 ± 21.66        | 17.18 ± 1.05       |
|                     |                  | Ubi Kuning  | 489.67 ± 56.83        | 13.79 ± 0.84       |
|                     | Water-deficit    | Roti        | 580.67 ± 21.67        | 14.56 ± 0.68       |
|                     | treatment (µm)   | Adira-4     | 485.33 ± 76.66        | 11.78 ± 0.68       |
|                     |                  | Ubi Kuning  | 589.33 ± 48.25        | 12.34 ± 0.53       |

**Note:** Means ± standard error within a column followed by the different letters in each column are significantly different at P≤0.05 by ANOVA
**Figure 3.** Stomata phenotypes in cassava abaxial leaf observed at 26 days after well-watered (left) and water-deficit (right) treatments. A-B, Roti; C-D, Adira 4; and E-F, Ubi Kuning

Stomata lengths of three genotypes were relatively persistent stomata length under well-watered and water-deficit treatments on days 26 and 45 (table 2). Stomata length alteration showed to correlate with the growth of several plants, such as broadleaf tree and herbaceous plants [14]. Meanwhile, the
stomata width significantly altered under water-deficit treatment after day 45 in all genotypes (table 2). After 45 days in water-deficit condition, Adira 4 showed more narrow stomata than Roti and Ubi Kuning (table 2; Figure 3). This may indicate the stomata width corresponded to water stress after 45 days by regulating the stomata pore aperture. Alteration in the stomata pore aperture could enhance the optimal uptake of CO$_2$ for photosynthesis and limit the water loss [6, 19, 26]. Alteration in the stomata dimension may be useful as one of the physiological markers for screening drought tolerance germplasm as shown in durum wheat [20].

3.2. Gene expression of reference genes and Aquaporin PIP2

To understand the molecular mechanisms regulating plant responses to water-deficit condition, the relative expressions of Aquaporin PIP2 (AQP) were observed in Roti, Ubi Kuning and Adira 4. First, two reference genes, i.e. $\beta$-tubulin (BTUB) and Manihot esculenta elongation factor 1-alpha (EF1-A), were analyzed for their stable expressions over all samples and experimental conditions. Results showed that BTUB had the smallest variation of expression levels than EF1-A, with a variation value of 1.48 (Figure 4). This variation value accounts for all genotypes and both water conditions. Therefore, the BTUB gene was selected as a reference gene to normalize AQP relative expression levels. Analysis and selection of stable expression of reference genes should be performed prior to the analysis of the targeted gene. This is important for controlling variables in quantifying targeted genes, including different amounts and qualities of samples, total RNA, and cDNA [27]. Expressions of reference genes may vary among different species, experimental treatments, accessions, organs, tissues, and cells [27, 28, 29]. In the experiment of assessing carotenoid gene expression using root-tubers of Adira 4 and Ubi Kuning, ZincFinger Protein gene showed to have more stability in gene expression than BTUB and EF1-A [29].

![Figure 4. Expression levels of $\beta$-tubulin (BTUB) and Elongation Factor 1-Alpha (EF1-A) in Roti, Adira 4, and Ubi Kuning under well-watered and water-deficit condition. Each boxplot describes variation in expression levels measured by Threshold cycles (Ct) of three cassava genotypes. SD describes the variation of the Ct values of each gene.](image)

Relative expressions of AQP showed that each cassava genotype had different AQP expression levels in both water treatments (Figure 5). In Adira 4, the expression levels of the AQP gene increased two-times at day 26 under water-deficit treatment compared with AQP expressions in well-watered treatment. After 45 days in water-deficit treatment, AQP expression levels continued to elevate up to three times higher than those expressed in Adira 4 plants under well-water treatment. In contrast, Roti,
which already had higher expressions of AQP under well-watered condition, showed a no significant reduction of AQP expressions in water-deficit treatment at day 26 and 45 (P>0.05). AQP expressions on 26th day under water-deficit treatment in Ubi Kuning only increased one-sixth more than that of expressions in well-watered treatment. However, 45 days after water stress treatment, AQP expressions reduced to the same levels as detected in well-watered condition. Different AQP expressions after water-deficit stress suggest that the molecular mechanisms underlying responses to water stress undergo differently among genotypes.

In this research, we have observed stomata phenotypes and AQP expression alteration due to water-deficit treatment in three cassava genotypes. Of all genotypes, Adira 4 showed different responses to water-deficit treatment, i.e., no significant stomata phenotypes alteration, but a significant elevation in AQP expressions (P<0.05). These suggest that a part of the adaptation mechanism to water stress conditions in Adira 4 was by regulating water permeabilities in cellular membranes using water channel proteins AQP PIP2. AQP PIP2 has been considered to be a more efficient water channel protein than other PIP protein families [30]. Similar responses have also been reported in Xenopus oocyte and Arabidopsis, which show an increase in water permeability under overexpression of the AQP PIP2 gene [21, 30]. Responses observed in Roti indicated that stomata phenotypic alteration was one of the adaptation mechanisms controlling the water stress environment. In addition to stomata phenotypic changes, Ubi Kuning may regulate its water channel proteins AQP PIP2 to control turgor pressure in the beginning phase of water stress condition.

![Relative expression levels for Aquaporin PIP2 (AQP) gene.](image)

**Figure 5.** Relative expression levels for Aquaporin PIP2 (AQP) gene. Blue, red, and purple bars represent averages of gene expressions from three biological replicates of each cassava genotype under well-watered, water-deficient at day 26, water-deficient at day 45 treatments, respectively.

### 4. Conclusions
Three cassava genotypes, Roti, Adira 4, and Ubi Kuning showed different responses to adapt water-deficit treatment. Alteration in stomata densities and length has not been observed in Adira 4, but significant expressions of water channel protein AQP PIP2 gene were observed as a response to the water-deficit condition. Roti possessed an adaptation mechanism to the water-deficit condition by regulating stomata phenotypes. Also, Ubi Kuning showed adaptation mechanisms to the condition by regulating stomata characteristics and water permeability. These results give a glimpse of adaptation mechanism in cassava genotypes, which were essential for selecting appropriate germplasms for cultivation under the unfavoured environment and for breeding aimed at cultivars with better adaptation in the water-deficit environment.
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