

## Morphological, physiological and molecular responses of Indonesian cassava to drought stress

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### Abstract

In Indonesia, investigations of drought-tolerant cassava by characterizing the morphological, physiological, and molecular responses have not been carried out. This research was aimed to characterize the morphological, physiological, and molecular features of 10 Indonesian cassava varieties (Adira 1, Malang 1, Cimanggu, Kaspro, Ketan, Litbang UK-2, Malang 4, Malang 6, UJ-3, and UJ-5) under drought stress. 30 days after planting, drought stress was applied by stopping irrigation of plants for 15 days. The plant height, root system, and wilting were measured as morphological responses of cassava. For physiological responses, the percentage of opening stomata, chlorophyll, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content were also investigated. Gene expression of *MeGBF3* and *MeMSD* was evaluated to analyze the response of candidate drought-tolerant genotypes. The studied parameters revealed that drought stress inhibits the growth of cassava. Some genotypes could not survive because the excessive content of H<sub>2</sub>O<sub>2</sub> would be toxic to plant cells and disturb the plant growth. The up-regulated gene expressions of *MeGBF3* and *MeMSD* has correlations with morphological and physiological responses of cassava to drought stress. Adira 1 and Kaspro are thought as drought-tolerant genotypes due to the morphological, physiological, and molecular responses.

**Keywords:** chlorophyll; gene expression; hydrogen peroxide; *MeGBF3*; *MeMSD*.

**Abbreviations:** H<sub>2</sub>O<sub>2</sub>\_hydrogen peroxide; ROS\_reactive oxygen species; SPAD\_soil plant analysis development.

### Introduction

Cassava as one of the fastest growing staple crops at the global level has been recorded to have an annual average growth far above 3% over the past decade (Alesiani et al., 2016). After two decades of uninterrupted growth, cassava production is expected to experience a slight contraction in 2017, down to 278 million tons (FAO, 2017). In cassava, drought stress can reduce tuber biomass. It is estimated that stress drought is the most detrimental environmental pressure of plants, contributing more than 70% of the potential loss of agricultural products worldwide (Turyagyenda et al., 2013).

Drought tolerance affects several processes of developmental, morphological, and physiological plants. The response of cassava is followed by dehydration through root system, stomata closure in dry air, decay of old leaves (Kiriga et al., 2016) and reduction in the opening of the stomata (Simova-Stoilova et al., 2016). A current research in Indonesia, indicated that drought stress reduces the number of branches, shoot dry weight and the number of chili (Ichwan et al., 2017), and reduces plantlet height, number of leaves, number of roots and proline content of Batulegi rice, Sigambiri Putih and Sigambiri Merah (Nurhayati et al., 2017), and *Pueraria javanica* (Sinamo et al., 2018).

The limited number of molecular studies has ranked the library as a tag that is stated as normal from cassava under drought stress (Lokko et al., 2007). Turyagyenda et al. (2013) identified two genes (*MeGBF3* and *MeMSD*) as candidate drought-tolerance genes in Ugandan cassava. However, the analysis of the expression of Indonesian cassava genes under drought stress has not been done.

In Indonesia, the studies of drought stress in cassava are limited to morphological and physiological responses. A molecular approach is needed to find out several genes that play roles under the pressure of drought. However, the research on molecular responses in Indonesian cassava under drought stress has never been done. This study is used to characterize the morphological, physiological, and molecular responses of Indonesian cassava to drought stress. This result uses three parameters (morphological, physiological, and molecular) to select drought tolerance cassava. Furthermore, the purpose of this study is to determine candidates as drought tolerance cassava by gene expression analysis.

## Results and discussion

Drought stress can inhibit plant growth in each phase. Plants need to develop several defense mechanisms to maintain water balance in plant cells. This mechanism causes changes in morphological and biochemical processes in plants. Plants that are resistant to drought stress require the ability to survive in severe drought conditions (osmotic).

### Morphological responses

After 15 days of treatment, 10 Indonesian cassava showed different responses to control conditions and drought stress treatment. Cassava in the control conditions has a higher level of growth parameters such as plant height, number of leaves, percentage of opening stomata and wilting of cassava under drought stress (Table 1). The cassava plant height under drought pressure is reduced compared to control cassava: Adira 1 (91%), Malang 1 (85%), Cimanggu (88%), Ketan (60%), Kaspro (79%), Litbang UK-2 (74%), Malang 4 (96%), Malang 6 (78%), UJ-3 (63%), and UJ-5 (77%) (Supplementary Fig. 1). Drought stress also inhibits plant height significantly in onion, poplar, and rice (Paul et al., 2018).

Plant cells that grow under the pressure of drought will limit growth to maintain the balance of water in the cell and that causes several growth barriers to plants. So that, the drought stress significantly affects plant height and number of leaves. 10 Indonesian cassava under drought stress also has a lower number of leaves than cassava under control conditions. The percentage reduction in the number of leaves under control and drought conditions was Adira 1 (73%), Malang 1 (46%), Cimanggu (82%), Ketan (50%), Kaspro (77%), Litbang UK-2 (67%), Malang 4 (93%), Malang 6 (56%), UJ-3 (72%), and UJ-5 (69%) (Supplementary Fig. 1). Water deficits causes a reduction in water accumulation and plants must adapt to maintain water to avoid dehydration.

Decrease in plant water level will decrease turgor cell level. The cell extension will be limited, and the water content in the cell will be balanced. Stomatal closure is the most important way of a defense mechanism (Hu et al., 2017). But the stomata closure is not strong enough to avoid loss of water in the leaves. All 10 Indonesian cassava in this study showed reduction in stomata opening under drought stress, compared to control conditions (Table 1). Some varieties such as Adira 1 (83%), Cimanggu (95%), Kaspro (89%), and Malang 4 (98%) have a slight reduction in the percentage of opening stomata (Supplementary Fig. 1). The reductions aims to maintain the balance of water in the cell. Each plant has a different ability to regulate stomata conductivity in response to drought stress (Salazar-Tortosa et al., 2018).

Plants respond to drought stress based on leaf anatomical function (Nelissen et al., 2018). The reducing water levels in plants causes a reduction in turgor between cells. In this case, plants under drought stress will show wilting as a morphological response (Supplementary Fig. 1). Among the 10 Indonesian cassava, Adira 1 and Cimanggu were not classified in the wilting category. While Kaspro, Ketan, Litbang UK-2, Malang 1, Malang 4, Malang 6, UJ-3, and UJ-5 showed wilting signs and even some leaves began to dry up. Some plants with dry categories may find it difficult to maintain water balance in plant cells.

The root system is the first sensitive organ to the soil moisture. Root system among the 10 Indonesian cassava

under drought stress showed reduction in the number of roots. While in the control condition, some cassava varieties has a wide root system, root length, and root volume. Malang 1 (52%), Ketan (13%), Litbang UK-2 (31%), Malang 6 (65%), UJ-3 (33%), and UJ-5 (36%) have a higher reduction in the number of roots (Supplementary Fig. 1). These conditions will make the plant more difficult to absorb water, dehydrate and cannot survive because the plant cannot keep the water balance inside plant cells.

### Physiological responses

Apart from changes in morphological characters, there are also physiological changes in plants in response to drought stress. 10 Indonesian cassava showed different physiological responses between control conditions and drought stress treatment, such as in chlorophyll and H<sub>2</sub>O<sub>2</sub> content. Chlorophyll content in drought stressed cassava has a lower number than in cassava in the control conditions (Table 2). Some varieties such as Adira 1 (93%), Cimanggu (95%), Kaspro (95%), and Malang 4 (95%) showed a slight decrease in chlorophyll content (Supplementary Fig. 2). The lower content of chlorophyll will interfere with photosynthesis. This condition can make plants difficult to survive. The previous research on *Cajanus cajan* (L.), Millspaugh (Sujatha, 2014), and peanut (Liu et al., 2018) also showed that drought stress significantly affected the chlorophyll content. Some plants experience a deficiency of chlorophyll content synthesis under drought stress (Kałuzewicz et al., 2017).

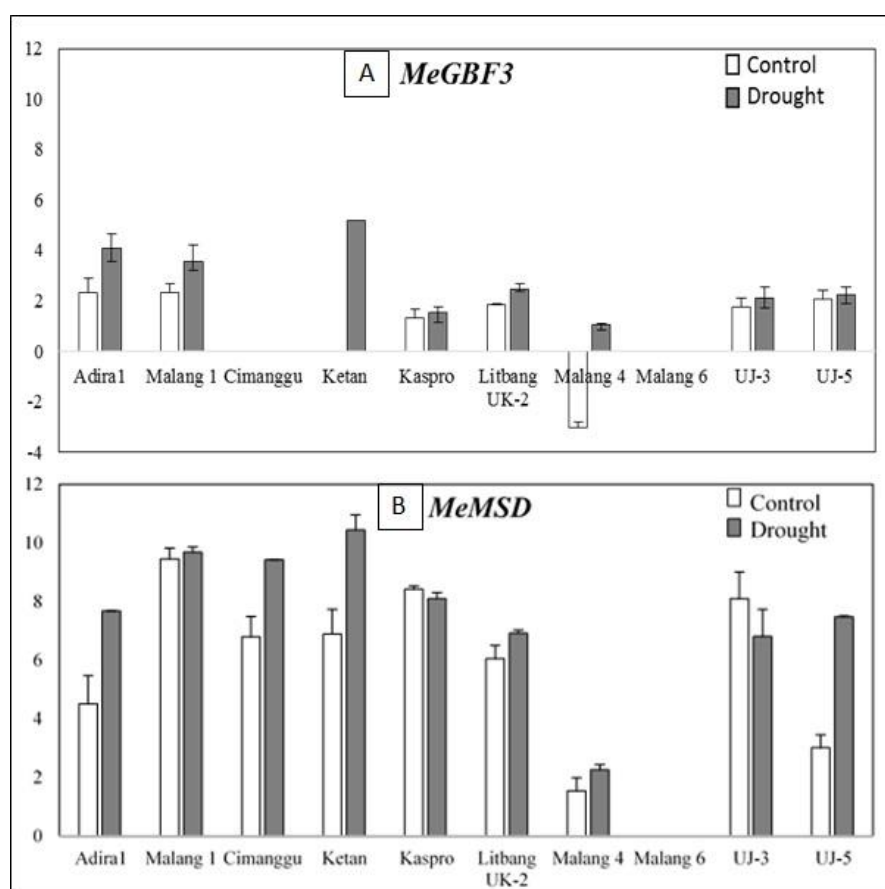
Conditions under drought stress encourage plants to produce reactive oxygen species (ROS). H<sub>2</sub>O<sub>2</sub> is ROS which plays an important role in the mechanism of defense against drought stress in plant cells. Excess H<sub>2</sub>O<sub>2</sub> content in plant cells can damage macromolecules, such as proteins, lipids, and nucleic acids (Wang et al., 2018). In 10 Indonesian cassava, Malang 6, UJ-3, and UJ-5 under drought stress had higher levels of H<sub>2</sub>O<sub>2</sub> accumulation than in control conditions (Table 2). While other varieties such as Adira 1 (59%), Cimanggu (51%), Malang 1 (63%), Kaspro (52%), and Malang 4 (39%) under drought stress have lower H<sub>2</sub>O<sub>2</sub> content than under control conditions (Supplementary Fig. 2). In a study by Khan et al. (2017), H<sub>2</sub>O<sub>2</sub> has been shown to affect activation or conditions in cellular processes. The lowest H<sub>2</sub>O<sub>2</sub> content can increase plant tolerance to various abiotic and biotic stresses.

### Role of MeGBF3 and MeMSD

Under the drought stress, plants produce several genes to control their metabolism such as *MeGBF3* and *MeMSD*. GBF3 is one of several G-box binding factors which are basic leucine zipper (bZIP) proteins. In Arabidopsis, *GBF3* is highly expressed in roots and is believed to be involved in regulation of alcohol dehydrogenase (ADH) through pathways that depend on ABA (Turyagyenda et al., 2013). Meanwhile, *MeMSD* encodes the enzyme manganese superoxide dismutase (MnSOD) which has a role in defense mechanisms under environmental stress. Superoxide is produced either in the matrix or on the inner membrane of the mitochondria facing the matrix. While most reactive species produced in mitochondria are superoxide anions, MnSOD quickly converts them to hydrogen peroxide.

**Table 1.** The effect of drought stress on plant height, number of leaves, percentage of opening stomata and wilting categories. Means followed by different letters are significantly different at  $p \leq 0.05$ .

Varieties	Plant height (cm)		Number of leaves		% Opening stomata		Wilting	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Adira 1	51.33 <i>ab</i>	39.66 <i>a</i>	28.33 <i>abc</i>	19.00 <i>ab</i>	99.96 <i>g</i>	99.96 <i>g</i>	Healthy	Healthy
Malang 1	52.16 <i>ab</i>	51.00 <i>ab</i>	30.67 <i>bc</i>	17.00 <i>a</i>	95.33 <i>g</i>	95.33 <i>g</i>	Healthy	Dry
Cimanggu	61.00 <i>bc</i>	42.33 <i>ab</i>	26.33 <i>abc</i>	21.33 <i>abc</i>	96.67 <i>g</i>	96.67 <i>g</i>	Healthy	Healthy
Kaspro	58.33 <i>abc</i>	42.00 <i>ab</i>	30.00 <i>bc</i>	17.00 <i>a</i>	85.67 <i>ef</i>	85.67 <i>ef</i>	Healthy	Wilting
Ketan	71.00 <i>c</i>	42.33 <i>ab</i>	24.00 <i>abc</i>	19.67 <i>ab</i>	77.67 <i>d</i>	77.67 <i>d</i>	Healthy	Dry
Litbang UK-2	54.33 <i>abc</i>	40.00 <i>a</i>	17.67 <i>a</i>	22.33 <i>abc</i>	95.00 <i>g</i>	95.00 <i>g</i>	Healthy	Dry
Malang 4	51.66 <i>ab</i>	49.66 <i>ab</i>	28.33 <i>abc</i>	17.33 <i>a</i>	94.33 <i>g</i>	94.33 <i>g</i>	Healthy	Wilting
Malang 6	60.33 <i>bc</i>	51.16 <i>ab</i>	32.67 <i>c</i>	24.67 <i>abc</i>	96.33 <i>g</i>	96.33 <i>g</i>	Healthy	Dry
UJ-3	47.33 <i>ab</i>	42.00 <i>ab</i>	21.67 <i>abc</i>	26.00 <i>abc</i>	87.00 <i>f</i>	87.00 <i>f</i>	Healthy	Dry
UJ-5	59.33 <i>bc</i>	51.66 <i>ab</i>	29.00 <i>abc</i>	19.33 <i>ab</i>	96.67 <i>g</i>	96.67 <i>g</i>	Healthy	Wilting



**Fig 1.** The gene expression of *MeGBF3* (A) and *MeMSD* (B) in Indonesian cassava under drought stress and control conditions.

**Table 2.** The effect of drought stress on chlorophyll and  $H_2O_2$  content in cassava. Means followed by different letters are significantly different at  $p \leq 0.05$ .

Varieties	Chlorophyll content (unit)		$H_2O_2$ content ( $\mu\text{mol g}^{-1}$ )	
	Control	Drought	Control	Drought
Adira 1	44.43 <i>gh</i>	40.26 <i>efg</i>	0.77 <i>f</i>	0.45 <i>b</i>
Malang 1	44.43 <i>gh</i>	32.36 <i>abc</i>	0.90 <i>h</i>	0.57 <i>c</i>
Cimanggu	41.36 <i>efg</i>	29.26 <i>a</i>	1.33 <i>j</i>	0.68 <i>de</i>
Kaspro	41.26 <i>efg</i>	34.43 <i>bcd</i>	1.11 <i>i</i>	1.11 <i>i</i>
Ketan	46.06 <i>h</i>	37.73 <i>de</i>	0.68 <i>d</i>	0.67 <i>d</i>
Litbang UK-2	44.10 <i>gh</i>	32.80 <i>abc</i>	0.79 <i>f</i>	0.71 <i>e</i>
Malang 4	42.16 <i>efgh</i>	31.23 <i>abc</i>	0.91 <i>h</i>	0.35 <i>a</i>
Malang 6	39.36 <i>ef</i>	31.70 <i>abc</i>	0.43 <i>b</i>	0.66 <i>d</i>
UJ-3	39.96 <i>efg</i>	30.16 <i>ab</i>	0.36 <i>a</i>	0.89 <i>h</i>
UJ-5	43.66 <i>fgh</i>	35.03 <i>cd</i>	0.57 <i>c</i>	0.85 <i>g</i>

**Table 3.** Primer in gene expression analysis.

Gen	Role	Primer
MeGBF3	Transcription factor and regulate alcohol dehydrogenase (Adh) by ABA	Forward: TGC ATC AAC TGT TGG GTG CG Reverse: ACC CAG AGC CAT GAG AAG GCT
MeMSD	Antioxidant/ROS/detoxification	Forward: ATG AAT GCA GAA GGT GCT GCA Reverse: GAA GGG CAT TCT TTG GCA TAC

Turyagyenda et al. (2013).

In this study, both *MeGBF3* and *MeMSD* were regulated under drought stress in Adira 1, Malang 1, Cimanggu, Kaspro, Litbang UK-2, Malang 4, UJ-3, and UJ-5 (Fig 1). *MeGBF3* is well-known for its role in ABA regulation and *MeMSD* has a role in antioxidant regulation/ROS/detoxification. Inhibition and reduction in plant height, number of leaves and roots, percentage of opening stomata, wilt, and chlorophyll content induced by ABA, *MeGBF3*. Meanwhile, H<sub>2</sub>O<sub>2</sub> content has a role that correlates to *MeMSD* activation. The regulation of gene expression is important in inducing several defense mechanisms in plants against drought stress. Some morphological and physiological responses to drought-tolerant cassava reflect the results of increased regulation of the expression of these genes.

## Materials and methods

### Plants and treatment design

The 10 Indonesian cassava varieties used in this study were Adira 1, Malang 1, Cimanggu, Kaspro, Ketan (as food source), Litbang UK-2, Malang 4, Malang 6, UJ-3 and UJ-5 (as industrial materials) originating from UPBSU Balitkabi, Malang, East Java, Indonesia. Cassava is grown in the greenhouse of Center for Development of Advanced Science and Technology, University of Jember, Jember, East Java, Indonesia with temperature during the day is around 25-40°C, while at night around 15-20°C.

Cassava cuttings (30 cm long) are planted in 20-L poly-bags using complete group design with three repetitions. Before planting the plants, each poly-bag is filled with 20 kg of soil (soil:compost 2:1 (v/v)). The cut of the plant is placed vertically in the middle of the soil of each poly-bag. Three plants per variety were included for each replication for each treatment. All plants were watered with 1 L of water every two days up to 60 days after planting. After 60 days of planting, the plants are treated with drought stress by applying no water for 15 days. In control plants, plants are watered 1 L every two days for 15 days.

### Morphological parameters

The measured morphological parameters include; plant height, number of leaves, root system, percentage of stomata opening, and plant wilt. This measurement was collected for three plants per variety from each repetition for each treatment after 15 days of drought stress. Plant height is measured with a meter. The total number of stomata, number of openings and stomata closure were calculated to see the percentage of stomata opening under the microscope observation. Plant wilt was analyzed based on research from Engelbrecht et al. (2007) which has been modified by Laban et al. (2013) in 3 scores (0 = dead, 1 = dry, 2 = withered, and 3 = healthy). The average value of morphological parameters in drought and control plants was

then calculated as a percentage to present a reduction in growth parameters in drought stress.

### Physiological parameters

The physiological parameters measured included chlorophyll and H<sub>2</sub>O<sub>2</sub>. These measurements were collected from three plants per variety from each repetition for each treatment after 15 days of drought stress, before leaf collection for gene expression analysis. Chlorophyll content in cassava leaves was measured by SPAD-502 meters (soil plant analysis development). Measurement using SPAD-502 meters aim to measure the greenness of leaves which correlates with chlorophyll content in the leaves. Analysis of H<sub>2</sub>O<sub>2</sub> must be done by collecting 0.1 gram of leaf samples, homogenized with to 1 ml of 0.1 % trichloroacetic acids (TCA). Then the sample was centrifuged at 10.000 rpm for 15 minutes at 4°C. The centrifugation was taken as much as 0.5 ml and added to 0.5 ml 10 mM phosphate buffer (pH 7) and 1 ml 1 M potassium iodide. Then, the solution was incubated at room temperature for 30 minutes. Absorbance is measured at a wavelength of 390 nm. Standard curve calibration is used as a standard in determining H<sub>2</sub>O<sub>2</sub> content (Weisany et al., 2012). The average physiological parameters in the drought and control plant are then calculated as a percentage to present a reduction in the parameters of *growth under drought stress*.

### Gene expression analysis

After 15 days of experiencing drought, fully inflated leaves were collected separately from three plants per variety from each replication in each treatment. RNA was isolated using RNeasy Plant Mini Kit Qiagen®. The RNA concentration is determined by spectrophotometry in Nanodrop (A260). The RNA samples were then converted to cDNA at RT-PCR using Reverse Tra Toyobo®. CDNA was used for gene expression analysis. The qRT-PCR showed in the standard system of Real-Time PCR using SYBR Evagreen Biorad®. QRT-PCR is run with three biological replications for each treatment for each variety. The 20 µL reaction volume consisted of the following: 10 ml of 2 x SYBR Evagreen I ready mix, 0.02 µL of passive dye, 1 µL each of the forward and reverse gene-specific primers, 2 µL of template cDNA (50 ng), and 5.98 µL of distilled, deionized water (ddH<sub>2</sub>O). The qRT-PCR conditions were as follows: initial denaturation at 95°C for 30 seconds, 40 cycles with denaturation at 95°C for 5 seconds, annealing at 55°C for 1 minute, and melting at 65°C for 5 seconds.

A reaction from qRT-PCR was normalized with the actin gene in cassava (primer: 5'TGCAGACCGTATGAGCAAG-3') as a calibrator in gene expression analysis. Meanwhile, *MeGBF3* and *MeMSD* are analyzed as library genes (Table 3). These genes have previously been reported from studies on varieties Uganda cassava varieties MH96/0686 and Nyalanda by Turyagyenda et al. (2013) which has been confirmed to

have a functional role in the stress of drought in cassava. A gene is significantly up-regulated or down-regulated when its expression on a treatment is higher or lower than that in a calibrator (standard/baseline).

### Conclusion

Adira 1, Cimanggu, Kaspro, and Malang 4 can be introduced as candidates of drought-resistant cassava, which are analyzed by lower percentage reduction in morphological and physiological responses. However, after gene expression analysis, Adira 1 and Malang 4 showed tolerance mechanisms through up-regulation of *MeGBF3* and *MeMSD*. In addition, characterization of morphological and physiological responses and more gene expression analyses are required to confirm these candidates are drought-tolerance cassava.

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### Supplementary material

The percentage reduction of morphological and physiological responses under drought stress in Indonesian cassava.

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