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The relationship between morpho-physiological changes and expression of transcription factors in NTT local rice cultivars as a response to drought stress

Yustina Carolina Febrianti Salsinha[1], Alfino Sebastian[2], Ekris Sutiyanti[1], Yekti Asih Purwestri[2,3], Didik Indradewa[4], Diah Rachmawati[1,*]

[1]Laboratory of Plant Physiology, Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
[2]Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
[3]Laboratory of Biochemistry, Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
[4]Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia
*Corresponding author: drachmawati@ugm.ac.id

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ABSTRACT Response by plants to drought occurs through a series of mechanisms that involve transcription regulation. This research was conducted to study transcription factors (TF) and physiological changes in the drought response of local rice cultivars from East Nusa Tenggara (Nusa Tenggara Timur, NTT) during drought stress. Using three NTT local rice cultivars (Boawae Seratus Malam (BSM), Gogo Jak (GJ), and Kisol Manggarai (KM)) and the fraction of transpirable soil water (FTSW) method with two treatment levels, FTSW 1 (control) and FTSW 0.2 (severe stress), we analyzed the TF expression of *OsDREB1A*, *OsDREB2A*, *OsWRKY45*, and *OsNAC6*. Based on the result, the highest level of TF expression occurred in the BSM, followed by the GJ and KM cultivars. Analysis of physiological characteristics showed an association between TF expression levels and physiological response, with the BSM cultivar showing high pigment levels, high proline content, and lower H_2O_2 levels. A linkage was also found in relation to water conservation, as indicated by the higher relative water content and cell membrane stability index in the BSM cultivar in contrast to lower electronic leakage and malondialdehyde percentage when exposed to drought. Based on the results, it can be concluded that the BSM cultivar can be considered as a drought-tolerant local cultivar according to morpho-physiological analysis. In this study, all NTT local rice cultivars showed a subtle upregulation of stress-responsive transcription factors *OsDREB1A*, *OsDREB2A*, *OsWRKY45*, and *OsNAC6* as responses to drought stress.

KEYWORDS drought; gene expressions; Nusa Tenggara Timur; local rice cultivars; transcription factors

1. Introduction

The East Nusa Tenggara (Nusa Tenggara Timur, NTT) region consists of several islands, including Timor, Sumba, Flores, Alor, Rote, and several other islands with diverse geographical conditions and dominated by dry climates (Hosang et al. 2018). Since the domination of dry climates, the increase of crop production in this area through drought-tolerant local rice cultivars cultivation needs to be solved. Drought tolerance is an adaptation mechanism that allows plants to survive drought stress without decreasing metabolic performance (Connor 2005).

Stress signals originating from the extracellular environment in the form of physical, chemical, and biological signals can affect plant changes at the metabolic, physiological and molecular levels. Signals originating from outside will interact cooperatively and synergistically to produce a final response through a series of signal transduction pathways (Chen et al. 2004). Signal transduction involves interactions between cells, intracellular and between individual plants (Memon et al. 1995).

In general, drought responses in plants occur through a series of signal transduction pathways, begins with drought signal perception by the receptor in membrane plasma proteins. These proteins interact and bind with extracellular molecules called ligands or elicitors that amplify through several steps. The amplification of stress signals downstream occurs through the induction of secondary messengers, such as inositol phosphate and ROS, which modulate the intercellular Ca^{2+} levels. Perturbation caused by the changes in Ca^{2+} level will be captured by calcium sensors, which then change its conformation to initiate several phosphorylation chain effects that lead to physiological responses and regulation of stress-

responsive gene expression (Xiong et al. 2002; Lata et al. 2015).

The signal transduction pathway plays a direct role in cellular metabolism during environmental stress. The drought signal transduction also led to the activation or deactivation of several genes involved in genetic regulation to synthesize specific proteins, enzymes, and metabolites. The regulations of stress response at the transcriptional and posttranslational levels undergo transcription factors activation and deactivation (Chen et al. 2016). The previous study (Riechmann and Ratcliffe 2000) showed that a large number of functional transcription factor ($\sim$ 1300) genes in Arabidopsis include families of specific DNA-binding domains like *NAC, ERF / AP2, Zn-finger, DOF, MYB, WRKY, bZIP*, and *HD-ZIP*.

Molecular studies of comparative transcriptomic analysis in abiotic stress responses show that the expression of the transcription factor is modulated by multiple stresses from the environment (Weiste et al. 2007). To find out a specific transcription factor involved in a specific stress response, it is necessary to consider several factors, including 1) family of the transcription factor, 2) the responses occur without stress induction, and 3) the function of transcription factors that must be validated in planta study through the transgenic approach, while for transgenic plants themselves, it is necessary to evaluate their stress tolerance level under drought stress conditions (Mattoo et al. 2015).

Several groups of transcription factors (TF) families are *AP2* / *ERF* consisting of *AP2* (Apetala 2), *RAV* (associated with *ABI3* / *VP1*), *DREB* (dehydration-responsive element-binding protein), *ERF* (Ethylene responsive factor), and several other groups. One example of *DREB* is *DREB1* and *DREB2*. The *DREB-2* control group is resistant to dehydration and salinity stress. The *OsDREB1A* gene under the control of the rd29A promoter showed increased resistance during dehydration with a high accumulation of antioxidant enzymes and proline (Yang et al. 2012).

Meanwhile, NAC TF regulates plant development, senescence, biotic response, and abiotic stress response. Overexpression of the *OsNAC6* gene in rice leads to drought and salinity tolerance in transgenic plants (Nakashima et al. 2007). The *OsNAC10* TF is expressed on roots with a specific promoter showing drought resistance, while *OsNAC45* TF is known to increase drought tolerance and salinity associated with induction of responsive genes against stress (*OsLEA3-1* and *OsPMI*).

Generally in the crop plants, WRKY TF is involved in a series of abiotic stress responses, one of which is that *OsWRKY11* under the control of a heat-inducible promoter shows increased drought stress, while *OsWRKY13* interacts with *OsNAC1* plays a role in the abiotic stress response pathway in plants (Qin et al. 2007). In this study, we examine the expression of *OsDREB1A*, *OsDREB2A*, *OsWKRY45*, and *OsNAC6* as transcriptional factors in regulating functional genes responsible for drought response through the examination of physiological changes in NTT local rice cultivars. To confirm the transcription activities of these transcriptional factors, several physiological characters, including pigment levels, proline contents, H_2O_2 content, relative water content, cell membrane stability index and ion leakage due to the damage caused by drought, had also been examined. The aim was to obtain an overview of the drought tolerance response in three potential local rice cultivars of NTT through its drought transcriptional factors expressions and physiological changes.

2. Materials and Methods

2.1. Plant Materials and Treatments

This research was conducted using three local rice cultivars of NTT as the drought-tolerant potential cultivars, namely Boawae Seratus Malam (BSM), Gogo Jak (GJ), and Kisol Manggarai (KM) based on the previous studies (Salsinha et al. 2021b,c). In the assessment of the drought tolerance of each cultivar under drought stress, the fraction of transpirable soil water (FTSW) method was used (Serraj et al. 2008) with two levels of stress, namely FTSW 1 (control) and FTSW 0.2 (severely stressed). The FTSW of each cultivar was determined by considering the number of the total transpirable soil water (TTSW) obtained by the difference between the fresh weight of the plant and the pot (W0) with the plant weight and the pot weight in the permanent wilting point (marked by constant weight) (Wt).

$$\text{TTWS} = W0(gram) - Wt(gram) \quad (1)$$

All plants were conditioned at FTSW levels ranging from 21 DAP (days after planting) to 42 DAP. To maintain the stability of each FTSW level, the amount of water kept in the pot (Pi) in each level were calculated using following the formula:

$$\text{Pi (ml)} = FTSW \times TTSW \quad (2)$$

Meanwhile, the weight of pots and plants that must be maintained in stable conditions (Wt) according to the respective FTSW level was calculated using the formula:

$$\text{Wt(gram)} = Pi - (TTSW - Wt) \quad (3)$$

Planting and cultivation were carried out with average rainfall between 250-350 mm, light intensity ranges from 5500 lx to 11000 lx during the day, and temperatures between 24-34 ºC from August to October 2020. The planting process begins with sowing rice seeds until they reach 21 days after imbibition (DAI) and then transferred to a pot with a diameter of 20 cm (1 kg capacity of soil: compost with a ratio of 3: 1, respectively). After reaching the age of 42 DAP, the plants were analyzed using the following parameters:

2.2. Molecular Analysis of Gene Expression

RNA was isolated from the leaves and roots of rice according to the protocol (FavorPrep™ Plant RNA Mini Kit 001-1 by Ping Tung Agricultural Biotechnology Park, Taiwan).

A total of 100 mg of root and leaf samples were crushed and extracted with FavorPrep™ FARB buffer containing ß-mercaptoethanol; the supernatant was homogenized with 70% ethanol and washed with wash FavorPrep™ buffer 1 and 2 containing 96% ethanol. The purity of the RNA was determined qualitatively using electrophoresis and quantitatively using a nanodrop spectrophotometer (Bichrome Nanodrop). RNA purification was done using the DNAse treatment with the DNAse I kit (Sigma Aldrich D5307 – Germany).

First-strand cDNA synthesis was carried out using Excel RT Kit II (SMOBIO Technology, Inc-Taiwan) according to the manufacturer's protocol with a total RNA of 500 ng. The purity of the cDNA synthesis results was tested using a nanodrop spectrophotometer. Gene expression analysis was carried out using semi-quantitative reverse transcriptase-PCR (sq-RTPCR) using ExcelTaq 5× PCR Master Dye Mix (SMOBIO Technology, Inc-Taiwan) with a composition of 1 µL cDNA template, 1µl forward primer, 1 µL reverse primer, 5 µL 5× PCR Master Dye Mix and 2 µL ddH_2O. The PCR program used consisted of the template denaturation stage and enzyme activation at 94 °C (2 min), the denaturation stage (94 °C (30 s), annealing at temperatures according to the Tm of each target gene primer, and extension at the temperature of 72 °C (2 min) and hold at 4 °C for 35 cycles (Table 1).

PCR amplification products were visualized by agarose gel electrophoresis (1%). The gel was prepared by dissolving 0.4 g of agarose in 40 mL of 1× TBE buffer and then mixed with 2 µL dye (FluoroVue). Before loading, 10 µL of PCR samples were mixed with 1 µL of DNA loading dye and loaded into wells compared to 5 µL of 100 bp DNA marker. The electrophoresis results were visualized using Gel Doc and analyzed using ImageJ to determine the percent density of each amplicon band resulted from PCR.

2.3. Physiological Characteristics Analysis

The pigments analysis was carried out by isolating photosynthetic pigments (chlorophyll and carotenoids) based method (Harborne 1984). A total of 0.3 g of leaves were ground with liquid nitrogen and homogenized with three ml of 80% acetone. Then, the absorbance of supernatants was measured at wavelengths of 470, 645, and 664 nm with a spectrophotometer with a blank of 80 percent cold acetone (GENESYS 10 UV Scanning, Thermo Scientific). Chlorophyll and carotenoid content were calculated by the following equation:

$$\text{Ch.a } (\text{mg} \cdot \text{L}^{-1}) = (12.7 \times A663) - (2.69 \times A645) \quad (4)$$

$$\text{Ch.b } (\text{mg} \cdot \text{L}^{-1}) = (22.9 \times A645) - (4.689 \times A663) \quad (5)$$

$$\text{Ch.total } (\text{mg} \cdot \text{L}^{-1}) = (20.2 \times A645) - (8.02 \times A663) \quad (6)$$

$$\text{Carotenoid } (\text{mg} \cdot \text{L}^{-1}) = \frac{(1000A4470) - 3,27(ChA) - 104(ChB)}{227} \quad (7)$$

Anthocyanin pigment was measured spectrophotometrically using 0.02 g and homogenized in 1 ml of extraction buffer containing 37% HCl, 1-propanol, and ddH_2O. Samples were incubated at 94 °C for 3 min and stored for 2 h in the darkroom at a temperature of 25 °C. After centrifugation at 13.000 rpm for 15 min, the absorbance of the sample was measured at 535, 650, and 720 nm with a blank of the extraction buffer (Lotkowska et al. 2015). Anthocyanin content was calculated by the following equation:

$$\text{Anthocyanin } (\text{mg} \cdot \text{g}^{-1}) = \frac{(A535 - 0,25) \times A650}{Fresh\ weight\ (g)} \quad (8)$$

Proline levels were measured by the method (Bates et al. 1973) using 0.25 g of leaves sample in 5 mL of 3% sulfosalicylic acid. One ml of the filtered supernatant was then reacted with 1 ml of ninhydrin acid and one ml of glacial acetic acid, then incubated at 94 °C for 1 h and cooled with an icebox. Proline was separated from the organic phase by adding 2 ml of toluene, and the absorbances were measured at 520 nm with the form of proline. The proline level in the sample was determined by comparing the results with a standard curve.

Determination of membrane peroxidation rate was carried out based on the content of malondialdehyde (MDA). A total of 0.25 g of leaves were crushed in liquid nitrogen and homogenized with 2.5 ml of 0.1% of trichloroacetic acid (TCA) solution according to method (Wang et al. 2019). The homogenate was centrifuged at 15.000 rpm for 20 min at 4 °C. One mL of supernatant was reacted with 4 ml of 0.5% of thiobarbituric acid (TBA) in 20% TCA heated at 95 °C for 30 min and cooled and the absorbance measured at wavelengths of 450, 532, and 600 nm. The blank solution is TBA 0.5% in 20% TCA. MDA content (9) was calculated by the following equation:

$$\text{MDA} = \frac{6.452}{6.42} \times [A523 - A600] - 0.559 \times A450 \times \frac{total\ extraction\ volume}{sample\ volume} \times fresh\ weight \quad (9)$$

TABLE 1 List of TF genes primers

No	Accession No.	Gene	Forward primer	Reverse primer	Tm (˚c)
1	AF300970.1	*OsDREB1A*	ATCAAGCAGGAGATGAGCGG	TGCCTCGTCTCCCTGAACTT	59.4
2	KU159749.1	*OsDREB2A*	GGCTGAGATCCGTGAACCAA	CGTGCTGTGGGACCATACAT	58.3
3	AB028185.1	*OsNAC6*	TCATGGCCGGTGAACTTTGA	GCACCATCTTTCTGCTGCTG	56.3
4	AY870611.1	*OsWRKY45*	CGGCAGTGTAGTGTCAGTCA	AGCTCCTTCCCCTTCTCCAT	58.3
5	EU650177	*Actin1*	AGCCACACTGTCCCCATCTA	TCCCTCACAATTTCCCGCTC	59.4

Hydrogen peroxide (H_2O_2) levels in the sample were analyzed using the method (Bouazizi et al. 2007) by homogenizing 0.25 g of leaves in 2.5 mL 0.1% TCA then centrifuged at a speed of 12.000 rpm and 4 °C for 15 min. The supernatant was taken as much as 0.5 mL and reacted with the reactants in the form of 50% TCA, 10 mM ferrous ammonium sulfate, and 2.5 M KSCN, then the absorbance was measured at 390 nm. The H_2O_2 content was determined by comparing it with a standard curve of H_2O_2 and H_2O_2 content (10) then calculated by the following equation:

$$\text{H}_2\text{O}_2 \text{ (ppm)} = \frac{(\frac{A390-a}{b}) \times volume\ test}{\frac{1000}{Fresh\ weight}} \quad (10)$$

Relative water content (RWC) was measured using 0.1 g (FW) leaf pieces taken from the third leaf and incubated in a tube containing 10 ml ddH_2O for 24 h under constant lighting. After incubation, plant weight was measured as turgid weight (TW), and each sample was dried in an oven at 70 °C for 48 h to determine the dry weight (DW), then the RWC percentage (11) was calculated using the formula (Mullan and Pietragalla 2012):

$$\text{RWC (\%)} = \frac{FW - DW}{TW - DW} \times 100 \quad (11)$$

The cell membrane stability index (CMSI) and the percentage of electrolytes leakage (EL) were measured by the method (Guo et al. 2020). A total of 0.1 g of leaves were cut to a size of 1 cm^2 and put into a tube containing 10 mL ddH_2O then incubated for 24 h. After incubation, ddH_2O was measured using an Electro-conductivity meter (EC Meter) to determine the initial EC value (EC_1). Leaf samples and ddH_2O were then incubated at 100 °C for 15 min and cooled to measure their EC as the final EC (EC_2). The CMSI (12) and EL (13) values are determined by equations as follow.

$$\text{CMSI (\%)} = [1 - \frac{EC1}{EC2}] \times 100 \quad (12)$$

$$\text{EL (\%)} = [\frac{EC1}{EC2}] \times 100 \quad (13)$$

2.4. Statistical analysis

The parameter measurement data of three rice cultivars treated with 2 levels of FTSW with 3 replications each were analyzed using One-Way ANOVA (IBM-SPSS Ver 25.0, USA). The level of data significance was further tested using the Duncan test at 95% confidence level ($p < 0.05$).

3. Results and Discussion

The cellular and molecular responses of a plant in the biotic and abiotic stress conditions occur through a series of mechanisms starting from signal perception. This process leads to the activation of several mechanisms related to plant defense in unfavorable environmental conditions. According to previous study (Xiong et al. 2002; Jain 2013), an understanding of this process in signal transduction will provide an overview of plant responses, especially in stressful conditions (low temperature, drought, and high salinity).

In general, signal transduction in plants is initiated by signal perception followed by activation of several second messengers which modulate Ca^{2+} ion levels and protein phosphorylation, which plays a role in direct cellular response or through activation of a series of transcription factors that control several genes in stress regulation (Xiong et al. 2002; Li and Liu 2016). Some of the transcription factors in plants with drought stress conditions include *OsDREB1A*, *OsDREB2A*, *OsNAC6*, and *OsWRKY45* (Yang et al. 2012).

Molecular studies have revealed several genes induced or upregulated by drought stress. However, the signal pathways responsible for this induction process are unknown. Several transcription factors (TF) act as promoters, including *DREB2A* and *DREB2B*, which are activated by osmotic stress and play a role in responsive genes in osmotic defense (Aroca et al. 2012).

Apart from the *DREB* gene, several other types of TF genes have vital functions in plant tolerance to drought stress, namely *OsNAC6* and *OsWRKY45*. A total of 140 types of *NAC* genes were identified in rice(Fang et al. 2008). One type of *NAC* gene that plays an essential role in rice response to stress is the *OsNAC6* TF. The *OsNAC6* is a type of TF that is responsive to drought stress and regulated by the presence of abscisic acid (ABA) (Nakashima et al. 2007; Jeong et al. 2010). Meanwhile, the other TF is *OsWRKY45*, which also reported increasing biotic stress resistance and drought stress resistance in Arabidopsis plants (Qiu and Yu 2009). *OsWRKY45* regulation is also influenced by endogenous ABA available in plants.

The expression level of several transcription factors that play a role in defense against drought stress is shown in Figure 1. Figure 1a showed the expression of TF *OsDREB1A* relative to *ACT1* in the roots and leaves of local NTT rice plants. BSM cultivar showed a higher expression level in leaf organs compared to other cultivars. Meanwhile, the GJ cultivar showed a higher expression level in the root organs than BSM and GJ. However, the analysis of two-way ANOVA showed no significant difference between the control and stress conditions in the GJ and KM cultivars and the organs analyzed ($p > 0.05$). Otherwise, BSM cultivars also showed no difference between stress and control conditions ($p > 0.05$), which indicated that the level of expression of this gene was not affected by drought.

Meanwhile, Figure 1b showed the level of *OsDREB2A* TF expression that was significantly different ($p < 0.05$) between each cultivar (BSM, GJ, and KM) treated with severe drought and control based on Two-Way ANOVA. The expressions of *OsDREB2A* and the other transcription factors of all cultivars tested were compared in 35^{th} PCR cycles (optimum cycles that show a significant difference in the band thickness between treatments). The optimization process in the 20^{th} and 30^{th} cycles did not show any

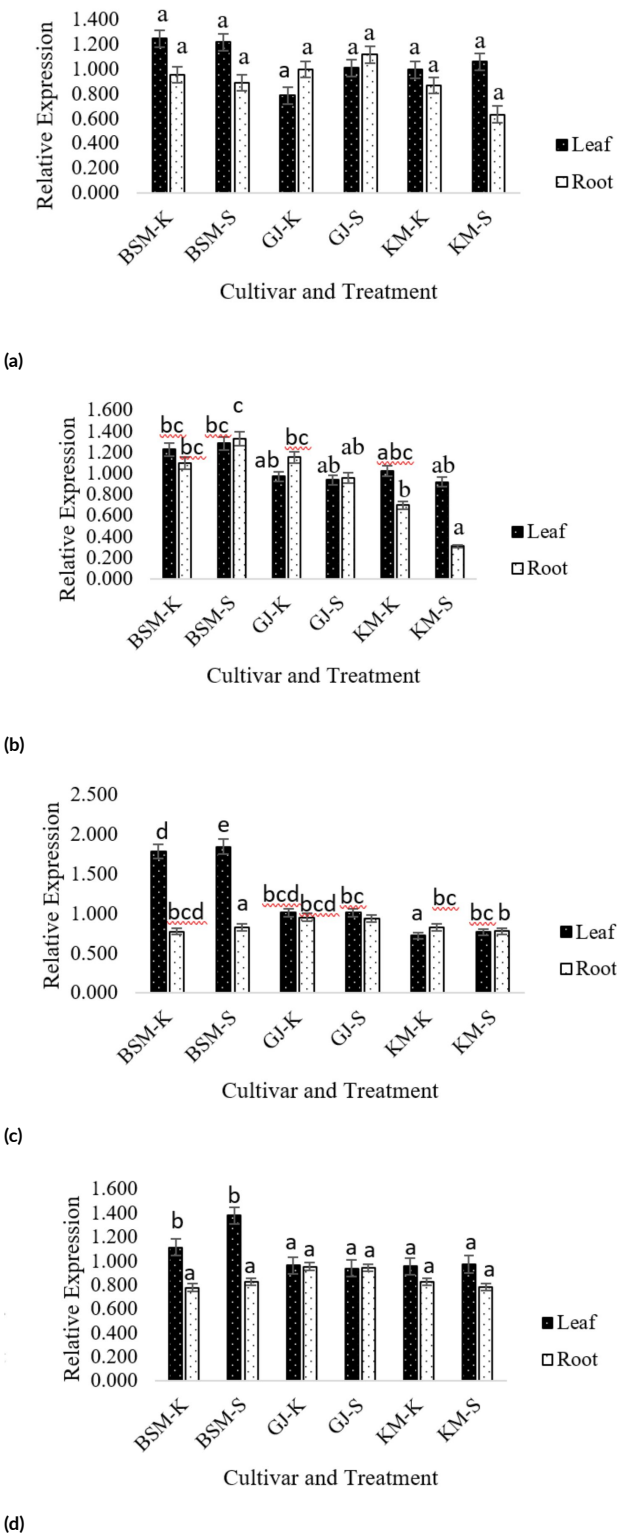

FIGURE 1 The relative expression of a) *OsDREB1A*, b) *OsDREB2A*, c) *OsWRKY45* and d) *OsNAC6* to Actin1 in the roots and leaves of NTT local cultivar rice: BSM= Boawae Seratus Malam, GJ = Gogo Jak and KM= Kisol Manggarai with the treatments of K = Control / FTSW 1 and S = Stress / FTSW 0.2. The mean (n = 3) followed by the same letter for each parameter shows no significant difference based on the Duncan test at the 95% confidence level.

difference in the band thickness of the electrophoresis results. In this 35th cycle, the measured expression was also significantly different statistically. However, one of the weaknesses in the PCR method with electrophoresis is that the value is determined semi-quantitatively and not based on the quantity of amplicon produced during PCR.

In Figure 1b, the lowest gene expression level was observed in KM cultivars (roots and leaves), while the highest level of TF expression was shown in BSM cultivars. Figures 1c and Figure 1d show the same expression pattern for the TF expression of *WKRY45* and *NAC6*. Based on Figure 1c and 1d, the expressions of TF in the leaves of the BSM cultivar was higher than in other cultivars and significantly different ($p < 0.05$).

TF regulation affects the activation of genes responsive to stress, especially drought, through a series of processes. Several responsive genes associated with the upregulation of transcription factors are responsible for osmotic, oxidative, and metabolic responses during the drought phase leading to structural and morphological changes associated with drought tolerance (da Silva et al. 2011; Salsinha et al. 2021a).

Figure 2 shows the differences in TF expression with differences in root and leaf organs in three NTT local rice cultivars under control (FTSW 1) and stress conditions (FTSW 0.2). The upregulation of some TF was confirmed by the changes in the morphology of shoots and roots parameters (Figure 3.). Based on Figure 3, during severe drought stress (FTSW 0.2), KM showed lower plant height (Figure 3a) compared to BSM and GJ ($p < 0.05$), while in the number of leaves (Figure 3b), BSM and GJ show a higher number of leaves with significance difference ($p < 0.05$) with KM. The BSM cultivar also showed higher shoot and dry weight while treated with FTSW 0.2 (severe drought stress) (Figure 3c and 3d). The changes in plant height, number of leaves, shoot, and dry weight appears in plants in response to drought stress. The more plant survives to drought, the more plants adapt with lower change of growth parameters than between control and severe drought treatment.

Meanwhile, the downregulation of TF was shown indirectly by KM cultivar with a higher reduction of morphological parameters during the drought phase (Figure 3). Indirectly, these changes may be related to the post-transcriptional regulation of each TF, which is responsible for metabolic and physiological changes during the drought stress treatment (Pandey and Shukla 2015; Wani et al. 2018).

Some of the physiological responses shown during drought treatment are the rate of photosynthesis or the assimilation process in leaves in terms of photosynthetic pigments. Photosynthetic pigments in the leaves are the main target of damage carried out by free radicals formed during the stress phase (Swapna and Shylaraj 2017). The primary photosynthetic pigments include chlorophyll a, chlorophyll b, and carotenoids are highly sensitive to physiological changes caused by drought (Phule et al. 2019; Salsinha et al. 2021c). Therefore, we measured the chloro-

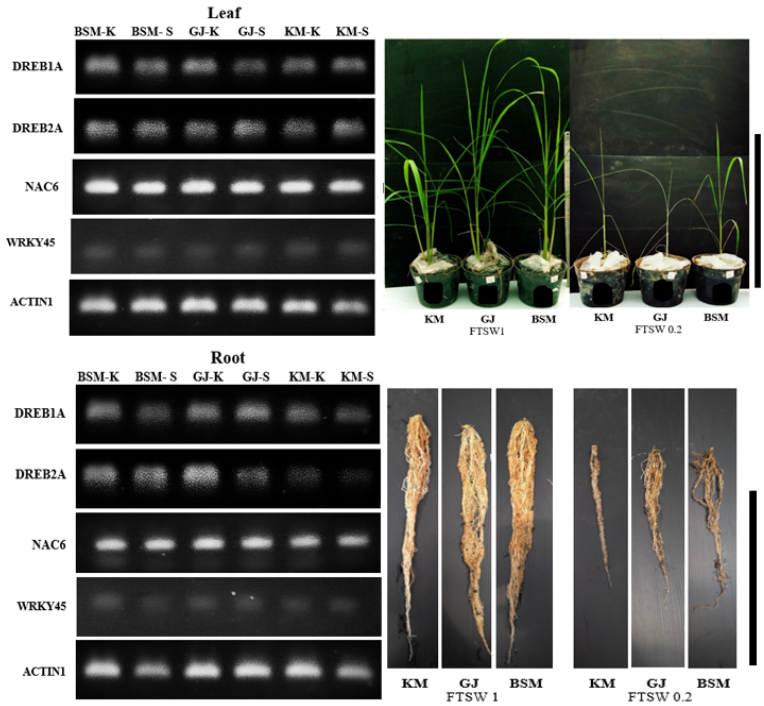

FIGURE 2 The expression levels of *OsDREB1A*, *OsDREB2A*, *OsWRKY45*, and *OsNAC6* relative to *ACTIN1* in the leaves and roots of NTT local cultivars: BSM = Boawae Seratus Malam, GJ = Gogo Jak and KM = Kisol Manggarai treated with K = Control / FTSW 1 and S = Stress / FTSW 0.2. Right bars: 60 cm., respectively.

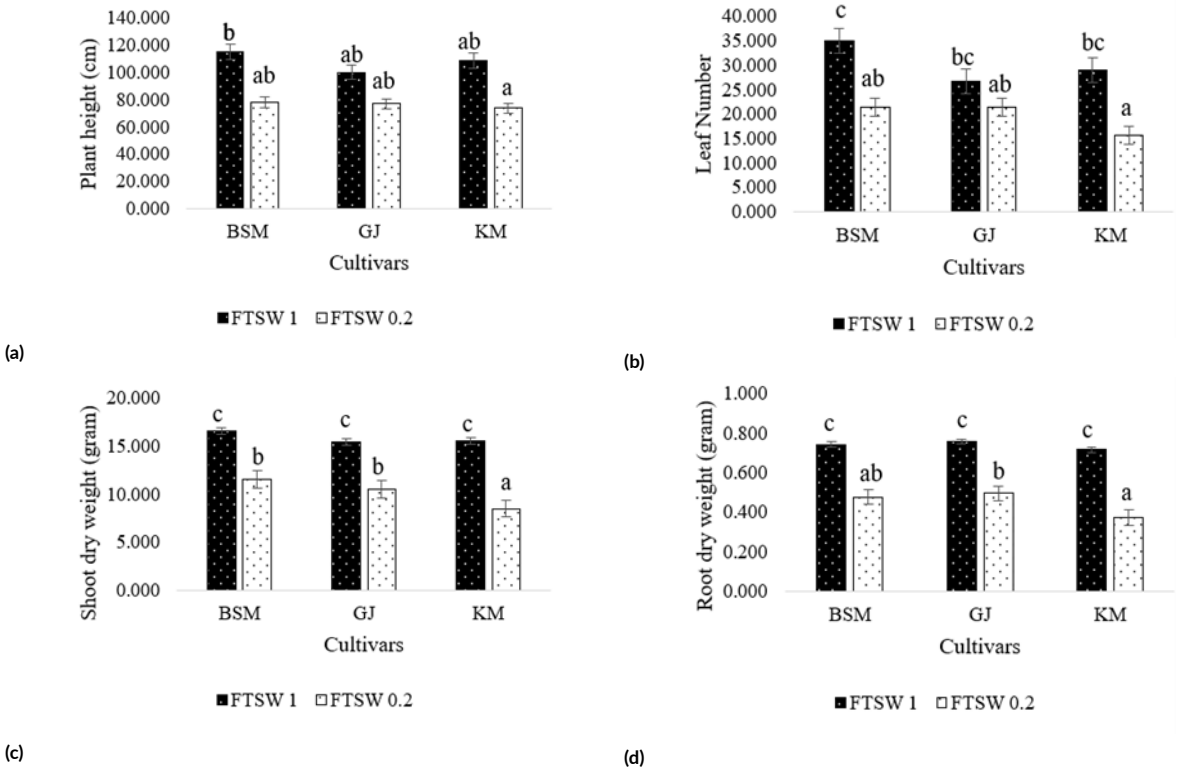

FIGURE 3 The growth parameters include a) plant height, b) leaf number, c) shoot dry weight and d) root dry of NTT local cultivar rice: BSM= Boawae Seratus Malam, GJ = Gogo Jak and KM= Kisol Manggarai with the treatments of K = Control / FTSW 1 and S = Stress / FTSW 0.2. The mean (n = 3) followed by the same letter for each parameter shows no significant difference based on the Duncan test at the 95% confidence level.

phylls and carotenoid contents of the three rice cultivars under control and drought treatment conditions (Figure 4).

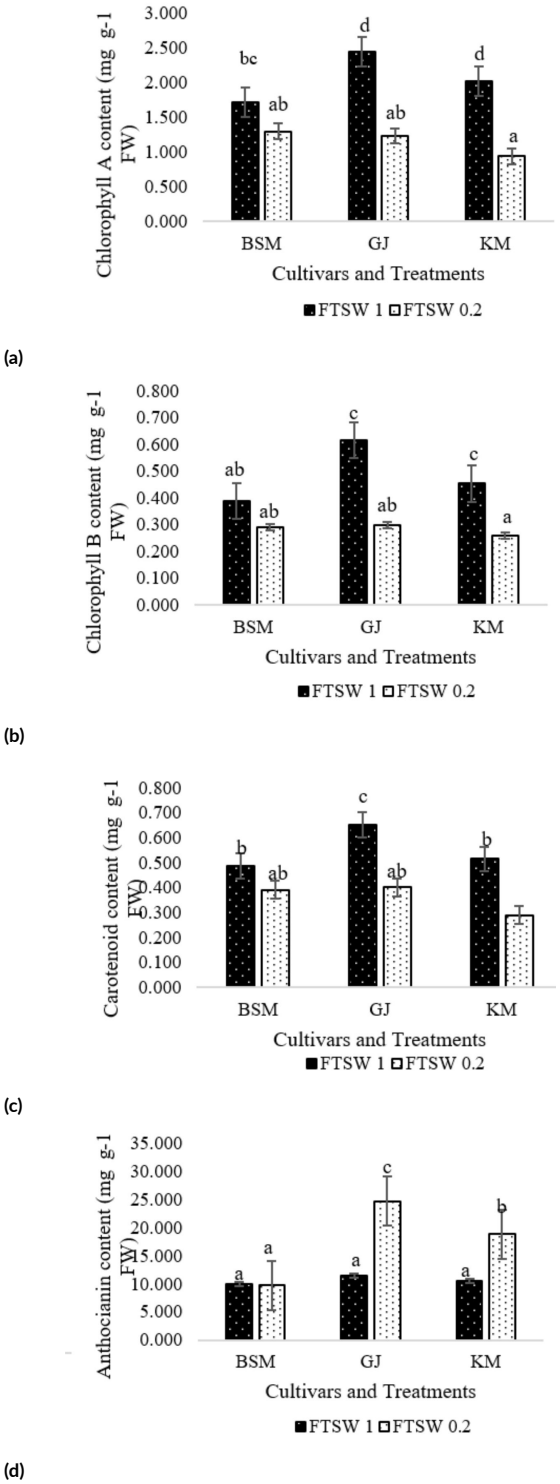

FIGURE 4 The levels of a) chlorophyll a, b) chlorophyll b, c) carotenoid and d) anthocyanins content in NTT local cultivars: BSM = Boawae Seratus Malam, GJ = Gogo Jak and KM = Kisol Manggarai treated with 2 levels of FTSWs: K =FTSW 1 and S = FTSW 0.2. The mean (n = 3) followed by the same letter for each parameter shows no significant difference based on the Duncan test at the 95% confidence level.

The data (Figure 4) showed that in drought stress, plants experienced a decrease in pigment levels, including chlorophyll a, chlorophyll b, carotenoids, and anthocyanins with significantly different values ($p < 0.05$ and $F > 4.34$) under control and stress conditions. However, notably, BSM cultivars showed no significant difference in the levels of chlorophyll b and anthocyanins ($p > 0.05$ and $F < 4.34$) between control and stress treatments, which showed no significant change during drought stress. This data also shows a response in the photosynthetic apparatus, which ensures the stability of photosynthetic pigment level and photosynthesis process even though the plants were exposed to drought conditions (Tiwari et al. 2010; Chaves et al. 2011).

Chlorophyll a, b and carotenoids are the main photosynthetic pigments of plants. Chlorophyll a present in Photosystem I and Photosystem II to maintain the stability of the photosynthetic process, as well as Chlorophyll b which functions to absorb sunlight and distribute it to chlorophyll for the photosynthesis process, while carotenoids act as photo-protectors, antioxidants, color attractants, and precursors of plant hormones in non-photosynthetic organs of plants (Bertolino et al. 2019; Maoka 2020). In general, drought stress can reduce chlorophyll and carotenoid concentrations in plants, but plants with high tolerance to drought show less dramatic reduction (Chaves et al. 2011).

The response of plants in maintaining the regular metabolic rate includes the photosynthetic process during the drought stress phase is closely related to the osmotic defense mechanism. The osmotic level ensures cell turgidity to support the metabolic processes. One of the defense mechanisms for plant osmosis occurs through the upregulation of genes responsible for synthesizing proline as an osmoprotectant. Proline is an amino acid responsible for preventing water loss from within cells and protecting the metabolic apparatus from ROS damage during drought stress (Filippou et al. 2014)

The result showed differences in proline levels in rice leaves and roots when exposed to control and drought stress. Based on Figure 5, in cultivar BSM and GJ, proline levels are induced by drought stress ($p < 0.05$), while in KM cultivar, drought stress caused a decrease in proline

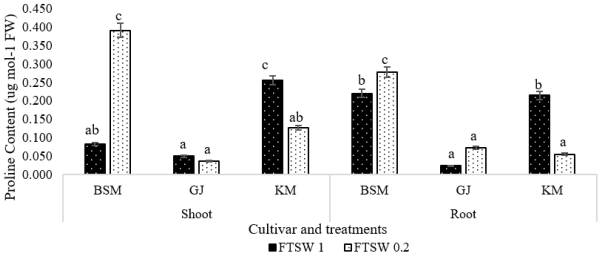

FIGURE 5 Proline levels in the roots and shoot in NTT local cultivars: BSM = Boawae Seratus Malam, GJ = Gogo Jak and KM = Kisol Manggarai treated with 2 levels of FTSWs: K = FTSW 1 and S = FTSW 0.2. The mean (n = 3) followed by the same letter shows no significant difference based on the Duncan test at the 95% confidence level.

level. In general, the higher the stress level, the higher the proline level produced by the drought-tolerant plant (Hayat et al. 2012). In the leaf organ, changes in proline levels were significantly different between control and stress treatment ($p < 0.05$), with the highest proline levels shown by the BSM cultivar.

Leaves as photosynthetic organs are closely related to plant biomass formation and overall energy fulfillment. The presence of osmoprotectant in leaves prevents cell damage and water loss within the leaf cells. The higher the proline level in the leaf organ, the more responsive the plant is to drought, which is closely related to a stable rate of photosynthesis during both the drought and control phases (Lehmann et al. 2010; Hayat et al. 2012).

Although the proline level was much higher in the root than in the leaf organ, apparently, there was no significant difference in proline content between the control and drought-stressed conditions in each cultivar in the root (Figure 5). This insignificant difference is possible because the root organ is the part that primarily experiences drought signal perceptions and is highly sensitive to the changes in water levels in the growing medium. The presence of high levels of proline in the root organs promotes a faster response when the plant is exposed to drought conditions, thereby avoiding the effects of excessive damage caused by ROS accumulation (Figure 5).

Previous studies have shown that photosynthetic organs have a higher level of gene expression responsible for proline synthesis (*P5CS* and *P5CR*) while catabolism gene (*PRODH*) in these organs is suppressed. Those *P5CS*, *P5CR* and *PRODH* genes were strictly regulated by TF (Figure 6), including *DREB1A*, *DREB2A*, *NAC* and *WRKY*. In contrast, proline breakdown occurs significantly in root organs, particularly in the meristematic zone (Ágnes Szepesi and Szőllősi 2018).

Some cultivars have a different defense response apart from the osmotic response. One of the most common is the oxidative response. Free radicals, often called reactive oxygen species (ROS), are very unstable and reactive. Free radicals can cause severe tissue damage. Therefore, in protecting themselves from free radicals, plants carry out oxidative defenses by forming antioxidant substances (Velázquez et al. 2003). This process is generally induced by TF activation in the pathway (Figure 6) of oxidative gene regulation, which leads to the synthesis of antioxidant enzymes and some non-enzymatic antioxidant components.

In the process, oxidation in plants is carried out through catalysis of ROS to H_2O_2 by the Superoxide dismutase (SOD) enzyme, followed by a cascade reaction of converting H_2O_2 into water and oxygen components in cells (Refli et al. 2015; Refli and Purwestri 2016). In this study, H_2O_2 was used as a component to measure the level of antioxidant activity in local NTT rice plants. Generally, drought-tolerant plants have a fast oxidative response so that ROS levels are marked by the lover content of H_2O_2.

Figure 7 shows rice H_2O_2 content under control and drought treatment conditions in response to drought conditions. All three rice cultivars experienced a significant increase in H_2O_2 content ($p < 0.05$). The H_2O_2 content relates to the radical oxidation activity when the

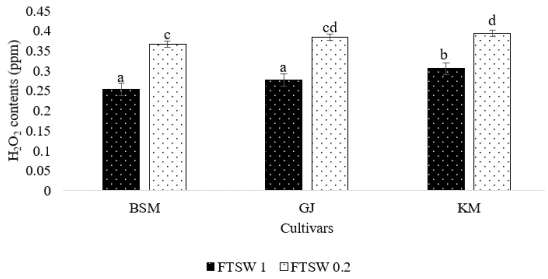

FIGURE 7 H_2O_2 contents in the roots and shoot in NTT local cultivars: BSM = Boawae Seratus Malam, GJ = Gogo Jak and KM = Kisol Manggarai treated with 2 levels of FTSWs: K = FTSW 1 and S = FTSW 0.2. The mean (n = 3) followed by the same letter shows no significant difference based on the Duncan test at the 95% confidence level.

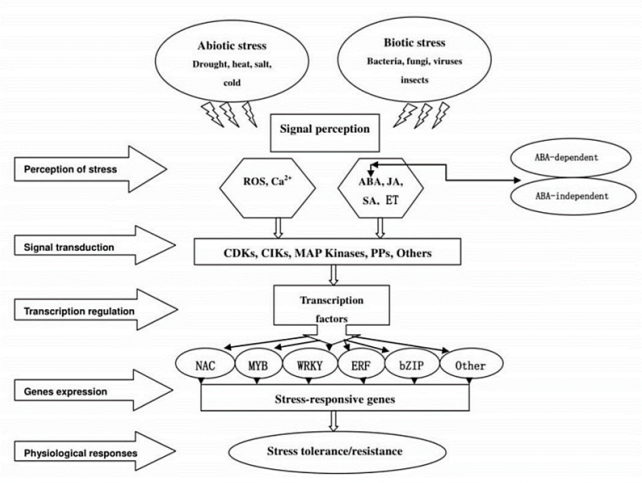

FIGURE 6 A schematic model of the signaling pathways involved *DREB*, *NAC* and *WRKY* TF (Baillo et al. 2019)

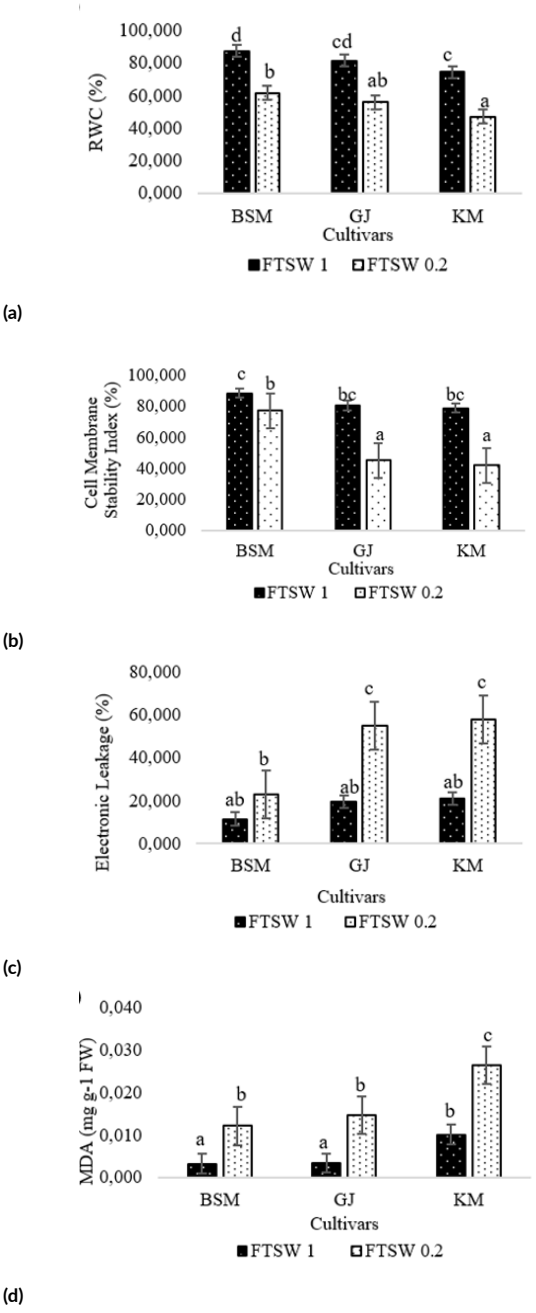

FIGURE 8 Physiological changes a) relative water content (RWC), b) cell membrane stability index (CMSI), c) electrolyte leakage (EL) and d) malonaldehyde content (MDA) of local NTT cultivars: BSM = Baoawae Seratus Malam, GJ = Gogo Jak and KM = Kisol Manggarai treated with K = Control / FTSW 1 and S = Stress / FTSW 0.2.

plants are exposed to drought conditions. During drought, the limitation of water in the photosynthetic apparatus causes a decrease in CO_2 absorption and inhibition of the NADPH reduction process from the light reaction, causing an oxygen-free reaction with other compartments in the cell (Aroca et al. 2012). This process can be neutralized by adding antioxidant components. The higher the response of a plant, the greater the antioxidant synthesized to reduce the levels of ROS, including H_2O_2 (Peters et al. 1939; Gechev et al. 2006).

The inability of a plant to adapt to water status change during the drought stress phase is caused by low antioxidant and osmoregulation responses. In this study, the TF expression level and the oxidative and osmotic regulatory pathways were also related to the ability of plants to respond to drought. Upregulation of TF that are responsible for this oxidative and osmotic process will lead to a more stable defense mechanism, thus preventing the effects of damage to cells and ensuring the resistance of a plant to drought conditions (Farooq et al. 2012).

In Figure 8, the result showed the difference in RWC (8a) between the three local NTT rice cultivars. Based on Figure 8, the highest RWC was found in cultivars of the BSM cultivar followed by the GJ and KM cultivar in control treatment with the percentage of 87.5, 81.5, and 74.4, respectively. Meanwhile, during drought stress, BSM, GJ and KM show the RWC percentage of 61, 55, and 46, respectively. This difference is due to the different responses to drought.

When compared with TF expression level, the mechanism of osmoprotectant synthesis and antioxidant response of BSM is relatively higher than other cultivars. In line with relative water content (RWC) (Figure 8a), the level of cell membrane stability index (CMSI) (Figure 8b) is higher in BSM ($p < 0.05$) than the other cultivar treated. The higher percentage of RWC and CMSI of BSM suggest that the osmoprotectant and antioxidant responses in BSM cultivar may play a role in maintaining cell integrity under drought conditions better than KM and GJ cultivars.

Under drought conditions, BSM cultivar showed a lower level of cell damage compared to other cultivars, as indicated by lower MDA levels (Figure 8d) ($p < 0.05$). Based on the data, in dry conditions, BSM accumulated less MDA and was able to regulate membrane integrity better, which was indicated by lower EL values (Figure 8d) compared to GJ and KM cultivars ($p < 0.05$). This indicates a higher drought tolerance response in BSM when observed from physiological changes.

4. Conclusions

Based on the research conducted, BSM cultivar can be considered as drought-tolerant local cultivar according to morpho-physiological analysis including proline, MDA, EL and RWC. In this study, as a response to drought stress, all NTT local rice cultivars show subtle upregulation of stress-responsive transcription factors *OsDREB1A*, *OsDREB2A*, *OsWRKY45*, and *OsNAC6*. In general, the transcription factors in drought defense regulatory pathways enhance oxidative and osmotic defense capabilities when exposed to drought stress.

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Authors' contributions

YCFS, DR conceived idea, YCFS, AS designed research methodology, YCFS, ES collected all data, YCFS, AS, YAP, DR interpreted the data, YCFS, DI reviewed literature, DI, DR analyzed the statistical data, YCFS, AS, ES, YAP, DI, DR wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare there is no competing interests.

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