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RESEARCH ARTICLE

Expression Analysis of *OsWRKY11* and *OsNAC2* Genes in New Malaysian Drought-tolerance Rice Cultivar MR303

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Abstract Local drought-tolerant rice variety MR303 (*Oryza sativa* spp. *Indica*) has higher tolerance towards abiotic stress while maintaining its high yield and grain quality. Expression of *OsWRKY11* has shown to be a positive modulator while *OsNAC2* is a negative modulator for drought-tolerance in *Oryza sativa* spp. *Japonica*. However, these transcription factor (TF) genes regulation are species-specific and its regulation may differ in our local rice variety. Thus, our study aimed to identify the relative expression of these genes and its effects on plant morphology and drought-tolerance capabilities. Our results on relative expression of *OsWRKY11* in the MR303 rice variety showed that under drought stress, this gene was highly expressed. This result was similar to previous findings in *Oryza sativa* spp. *Japonica*. However, for *OsNAC2* gene expression, our results contradict with previous findings where under drought stress, this gene was also highly expressed instead of downregulated. These results suggest that our local rice variety may have different gene regulation under drought stress compared to other rice varieties. Proline assay showed that proline contents in drought-treated plant has increased 10 times compared to control which associated with drought-tolerance activities. Further studies may be conducted to gain better understanding on the roles of these genes in regulating drought-responsive genes in the local variety.

Keywords: Transcription factor, *OsWRKY11* gene, *OsNAC2* gene, MR303 rice, proline.

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Introduction

Rice is considered as one of the plants that susceptible to abiotic stress due to its small root system, thin cuticular wax, and swift stomatal closure [1]. Rice commonly suffers multiple environmental stresses like drought, salt and extreme temperatures during their lifespan and they have evolved impressive mechanisms at molecular, biochemical, physiological and metabolic levels in order to cope with the diverse condition [2]. At molecular level, an effective and timely signaling network is initiated upon perception of external stress signals. This signaling network was programmed by the expression of a large set of stress-responsive genes via synergistic action from different types of transcription factors (TFs) in both temporal and spatial manners [3]. In the past decades, a few numbers of TF families have been recognized and further characterization of these TF families demonstrated that its plays an important role in plant abiotic stress response [2, 4]. These TFs promotes and activates stress related genes in different stress regulated pathways.

Various omics approaches had been conducted to identify important player among large families of TF in regulating broad range of gene expression to help plant adaptation towards drought stress [4]. Some of important TF families that have been identified are *AP2/ERF*, *bZIP*, *NAC*, *MYB*, *MYC*, *Cys2His2*, zinc-

finger, and *WRKY* families [5-8]. *WRKY* and *NAC* TF are amongst the most crucial TF families in regulating plant reactions towards drought stress and numerous reports have been done to elucidate the correlation of these TF family members and drought-tolerance in plants [9, 10].

WRKY TFs are members of the *WRKY-GCM1* superfamily of zinc finger TFs characterized by a conserved N-terminal WRKYGQK terminal in conjunction with C-terminal zinc-finger-like motif [11, 12]. Under stress, *WRKY* genes generally bind to W-box domain or W-box sequence (TTGACT/C; *cis*-acting elements), resulting in the expression of downstream target stress-responsive genes [13]. Numerous studies have been conducted on *WRKY* TF genes from model plant such as Arabidopsis [14], rice [15] and wheat [16] and non-model plant such as orange (*Poncirus trifoliata*) [17] and rapeseed (*Brassica napus*) [18] in response towards drought stress. Majority of the studied species shows that *WRKY* TF genes were up-regulated upon induction from drought stress.

Another TFs gene family is *NAC* TF genes. *NAC* proteins are among the largest plant-specific TF superfamily that play critical roles in regulating plant stress response. Extensive studies assisted by the availability of plant genomic sequences have identified 138 NAC genes in *Arabidopsis thaliana*, 170 in rice, 71 in grape (*Vitis vinifera*), 145 in citrus (*Citrus sinensis*), 269 in soybean and 280 in tobacco [19]. The *NAC* proteins are characterized with a conserved region, called *NAC* domain which located at their N-terminals whereas a highly divergent located at C-terminus [20]. A large number of the *NAC* TFs have been identified for their roles in biotic and abiotic stress responses [21, 22]. In transgenic rice plants, several *NAC* TFs were involved in regulating ABA-mediated signalling pathway as demonstrated by the ABA hypersensitivity response, upregulated expression of ABA-responsive stress-related genes, ABA biosynthesis-related genes and increased the endogenous ABA level [21].

WRKY and *NAC* TF gene expression have been proven to be associated with drought-tolerance but these TFs are species-specific [23, 24]. Thus, the effects of these TFs towards gene regulation may vary in our local rice varieties. Majority of conducted researches and database available were constructed using *Oryza sativa L*. ssp. *japonica* as model plant. In Malaysia, almost 95% of rice seeds available for commercial field plantation were generated by Malaysian Agricultural Research and Development Institute (MARDI) [25]. Seeds from cross breeding program of *Oryza sativa L*. ssp. *indica* are one of the high demand rice varieties from agricultural sectors and industries due to its high yield and grain quality [25]. Some studies has shown that the number of same family gene could differ between *Oryza sativa L*. ssp. *japonica* and *Oryza sativa L*. ssp. *indica* eventhough they were classified in similar group species [26]. Other than that, Malaysia is also a tropical country with sunny skies and humid rainfall throughout the year. Thus, our variety might differ in term of gene regulation due to the difference environmental factors. Our rice variety did not have to face extreme heat or cold due to change of seasons.

Generation of new commercialize *Oryza sativa L*. ssp. *indica* cv MR303 through cross-breeding on 2018 by MARDI has open an opportunity for us to further studies the effect of TFs in regulating drought stress on local variety. MR303 variety has been claimed to have higher yield and less susceptible to environmental stress such as drought and unfertile soil [27]. Thus, we can observe the relative expression (RE) of targeted *WRKY* and *NAC* TF genes correlation towards drought stress in the drought-tolerance variety. *OsWRKY11* and *OsNAC2* expression will be use as our marker for this study.

Overexpression of *OsWRKY11* in transgenic rice showed reduced leaf wilting after 8 days of drought treatment compared with wild-type plants [28]. After 5 days of re-watering, the transgenic rice has better rate of recovery compared to the wild-type plant. Within the same study, a drought tolerance assay was also conducted on suppressing *OsWRKY11* through gene knock-down. After 5 days of re-watering, *OsWRKY11* suppression cause an opposite effect towards plant growth where a lower rate of recovery from wilting was observed. Thus, this confirmed that *OsWRKY11* genes act as positive modulator in response to drought-stress and activating-drought-tolerance genes [28]. Another drought-related TF, *OsNAC2* was strongly induced by osmotic stress and abscisic acid. Overexpression of *OsNAC2* in rice cause the decrease in drought-tolerance trait [29]. 2-weeks without watering cause transgenic line overexpressing *OsNAC2* turned yellow and wilted. Re-watering of the wilted plant also showed significantly lower rate of survivability compared to the wild-type. However, suppression of *OsNAC2* showed positive grow even without watering for 2 weeks. In addition, re-watering of the plant after the drought treatment also showed higher survival rate when compared to the wild-type and overexpression *OsNAC2* rice [29]. These results suggest that *OsNAC2* act as negative modulator for drought tolerance in rice.

Both of previous studies were conducted in *Oryza sativa L*. ssp *japonica* which have an ecotype of Nipponbare. Thus, we will use both of these genes, *OsWRKY11* and *OsNAC2* as markers to observe the regulation of the same genes in our local variety MR303. Besides that, we will also observe the

growth of MR303 variety under normal and drought condition followed by proline assays to quantify the proline content of the rice plantlets. Proline accumulation and metabolism are positively associated with abiotic stress tolerance in plants [30]. This amino acid is one of the commonest plant osmolytes that significantly increase in response to water deficit, high salinity or other stressful conditions has been many plant species [31].

Materials and Methods

The workflow of the experiment was shown in Figure 1. Rice seeds obtained were grown for 3 weeks before the drought-treatment was started. The plantlets were exposed to drought condition until fully wilt with the plant height recorded every week. RNA was extracted from the leaf at week 5 for relative expression analysis. RNA was converted into cDNA before RT-PCR was conducted. Drought-tolerance assay was also conducted by measuring the proline content as an indication of drought-related activities.

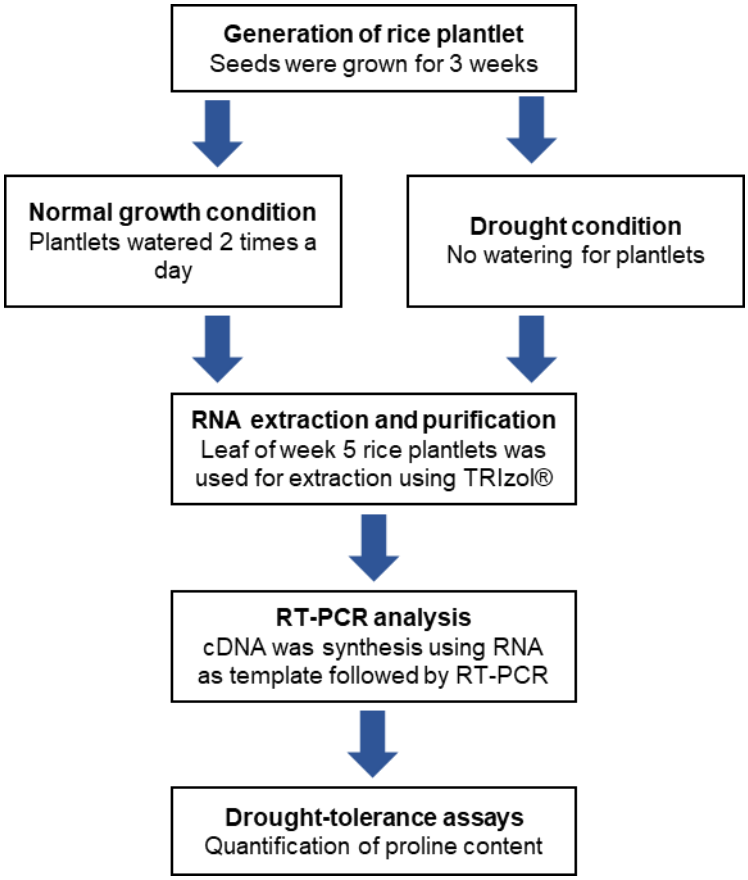

Figure 1. Workflow for relative expression analysis of *OsWRKY11* and *OsNAC2* in MR303 rice

Plant material, germination and treatment

Oryza sativa L. ssp. *Indica* cv MR303 seeds were obtained from MARDI Seberang Prai, Pulau Pinang. Rice seeds were surface-sterilised and stratified in the dark at 4°C for 3 days before seed were sown on soil. Sown seeds were germinated in a condition with temperature around 27°C during the day and 25°C during night time with humidity between 70% to 90% throughout the day. Seeds were also watered 2 times a day for three weeks until the seeds germinated into plantlets with 3 to 4 leaf each. At this stage, the plantlets were separated into two groups. The first group were labelled as control and watering were continued as usual, whereas the second group were labelled as drought and watering was permanently stop until the plant wilt [2].

Growth measurement analysis

For plant growth measurement, at least 12 plantlets height were measured every week starting from week 1 until week 7 after sowing. Photographs of plantlets were taken using a digital camera with a standard 15 cm ruler for scaling. Acquired photograph were processed using ImageJ 1.8.0 [32] to set the scales using pixels of the images with reference to the ruler. Statistical analysis was later conducted using t-test in Microsoft Office Professional 2019 to determine the significant difference between control and drought-treated plantlets every week.

RNA extraction and purification

For RNA extraction, leaf samples were harvested at week 5 for both control and drought-treated group. RNA extraction was conducted based on guanidine thiocyanate technique [33] TRIzol® reagent (Invitrogen, USA) with some modification. Frozen leaf samples were crushed in mortar before suspended in TRIzol reagent. Then it was heat up at 37°C for 2 minutes followed by 5 minutes incubation in room temperature. Sample was then centrifuge at 12000 rpm for 10 minutes at 4°C. Aqueous phase was pipet into new tube and chloroform was added to the tube. Tube was vortex and incubated in room temperature for 3 minutes before it was centrifuge at 12000 rpm at 4°C for another 20 minutes. After that, the aqueous phase was pipet out and mix with isopropanol with ratio 1:1 and left for 10 minutes in room temperature. Then, it was incubated overnight in -20°C.

After incubation, tube was centrifuge at 12000 rpm for 15 minutes in 4°C. Aqueous phase was removed and pellet in tube is washed with 80% ethanol. Then the tube was re-centrifuge at 7500 rpm for 5 minutes at 4°C. Ethanol was removed and the pellet was air-dried for 5 minutes before dissolving the pellet in RNase free water. The RNA sample was then treated with TURBO DNA-free™ kit (Invitrogen, USA) for removal of DNA genome following the product manual. TURBO DNase buffer and DNase was added to the RNA sample and mixed gently. Then, it was incubated at 37°C for 30 minutes before adding DNase inactivation reagent. Then, the solution was incubated at room temperature for 2 minutes before centrifuge at 12000 rpm for 1.5 minutes. The RNA was then transferred into new tube. The quality of the RNA was accessed using Nanodrop Spectrophotometer (Thermo Scientific, United Kingdom) and gel electrophoresis (Applied Biosystem, USA).

cDNA synthesis and semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis

The semi-quantitative RT-PCR analysis were conducted to identify relative expression of *OsWRKY11* and *OsNAC2* by comparisons with the reference gene *OsActin*. cDNA was synthesized from 1 µg of total RNA to the final volume of 25 µL reaction mixture using RevertAid Reverse Transcription kit (Thermo Scientific, United Kingdom) according to manufacturer's protocol [34]. After generating the cDNA strand, RT-PCR was carried out using thermocycler with the following program: 95°C for 5 minutes, 40 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72 °C for 30 seconds. The PCR product was visualized on an agarose gel and could be quantified in comparison to the reference gene. The primer pairs used in this study were as follow: *OsActin* (Genbank accession: XM469569) with 5'-TCCATCTTGGCATCTCTCAG-3' (forward) and 5'-GTACCCGCATCAGGCATCTG-3' (reverse), *OsWRKY11* (Genbank accession: Os01g43650) with 5'-AGCCCAGAGAGAAAGCTGAG-3' (forward) and 5'-TATGGGCTGTTCTTGACTGC-3' (reverse), *OsNAC2* (Genbank accession: Q8H0I4) with 5'-GTCGTCCGTCGTCCTCCCTC-3' (forward) and 5'-CTGCCGTCGGGTTAAAGAACTG-3' (reverse).

All PCR products were subjected by electrophoresis on GreenSafe-stained gels. The gel was then scanned using BioDoc-It™ imaging system (UVP, United Kingdom). The band quantification was carried out by measuring pixels density using ImageJ 1.8.0 [32]. The measurement resulted in band intensity as the expression unit. The ratio between the *OsWRKY11* or *OsNAC2* and *OsActin* was calculated to normalize for initial variations in sample concentration. Relative expression of target genes against internal control was calculated by the equation: RE = expression unit of target genes ratio/expression unit of internal control ratio [35].

Proline content analysis

For proline quantification, colorimetric assay method was used [36]. 100 mg of leaf were harvested from week 5 plantlets and grind in liquid nitrogen. Then, 0.5 mL of 3% sulfosalicylic acid was added and grind again before it was spin in microcentrifuge at maximum speed for 5 minutes. Next, reaction mixture containing 3% sulfosalicylic acid, glacial acetic acid and acid ninhydrin in 1:2:2 ratio was prepared in a new tube. 100 µL of supernatant from grind samples were added to the reaction mixture and incubated in heat block at 96ºC for 60 minutes. The reaction was then terminated by putting the hot tube into ice. 1 mL of toluene was added after the tube has cooled down and leave for 5 minutes. The chromophore of the solution was transferred into new tube and measured in a spectrometer at 520 nm with toluene as

a reference. The proline concentration was determined using a standard concentration curve and calculated on fresh weight basis.

Results and Discussion

OsWRKY11 and *OsNAC2* genes were found involves in enhancing drought-tolerance trait in MR303 rice variety. However, the expression of *OsNAC2* were upregulated instead of its function as negative modulator during drought condition which is opposite from the previous study on different rice variety. Proline content quantification also showed a significant increase in proline which indicates high drought-tolerance related activities. Our results suggest that MR303 may have different TF genes regulation which enhance the rice tolerance traits when compared to other rice varieties. This fundamental information may help us to plan more efficient strategy and approach in improving our local rice variety drought-tolerance capability.

Differences in plant growth between MR303 rice grown in normal and drought condition

In this study, we grow our seed up until three weeks to ensure that our plantlets were fully grown and manage to carry out self-supporting mechanism such as photosynthesis and transpiration process. Rice seed development start with vegetative stage which begin from seed germination up until tillering stage [37]. During this stage, there are transition of rice to become autotrophic or self-supporting [38]. The self-supporting capabilities usually achieved when rice plantlet emerges the 3rd and 4th leaf. This stage was also known as pre-tillering stage, where the seminal root further develops [37]. Usually, before the emerging of the 3rd leaf, seedlings are largely dependent on the stored seed reserves for growth and development.

3-weeks old plantlets were divided into two group where one group was subjected to drought-stress while the other act as a control. 2 weeks without watering will impose drought stress in rice and usually symptoms of wilting can be observed at this time point [29]. The first two weeks of drought treatment does not show any significant changes in term of plant height and wilting symptoms between control and drought-treated plantlet. However, at week 6 or 3 weeks after drought treatment, MR303 drought-treated plantlets started to show significant changes in plant height and wilting symptoms like browning and leaf shrinking (Figure 2). There were 21.2% reduction in plant height from 28.95 ± 1.87 cm to 22.82 ± 1.03 cm. On week 7, drought-treated plant has fully wilt where most part of the plantlet have dried up and turn to brown colour. At this time point, the gap of plant height is more tremendous where 32.7% reduction was observed from 33.73 ± 2.63 cm to 22.70 ± 0.83 cm. There was also no significant increase in plant height starting from week 6. We can also observe the absolute growth rate of the drought-treated plant has decrease after week 3 and come to a complete stop after week 5. This indicates that without watering, no further increase in plant growth and plant slows down their growth rate until the plantlet found a new source of water. In contrast, the control plantlet shows increase absolute growth rate after week 4. This time point was aligned with the rapid growth during active tillering stage which contribute to the increment of absolute growth rate in the plant [37]. The MR303 variety also shows higher drought tolerance when compared to previous study on *Oryza sativa L.* ssp *japonica* where browning of plant was observed after 3 weeks instead of 2 weeks as reported in the Nipponbare cultivar [29].

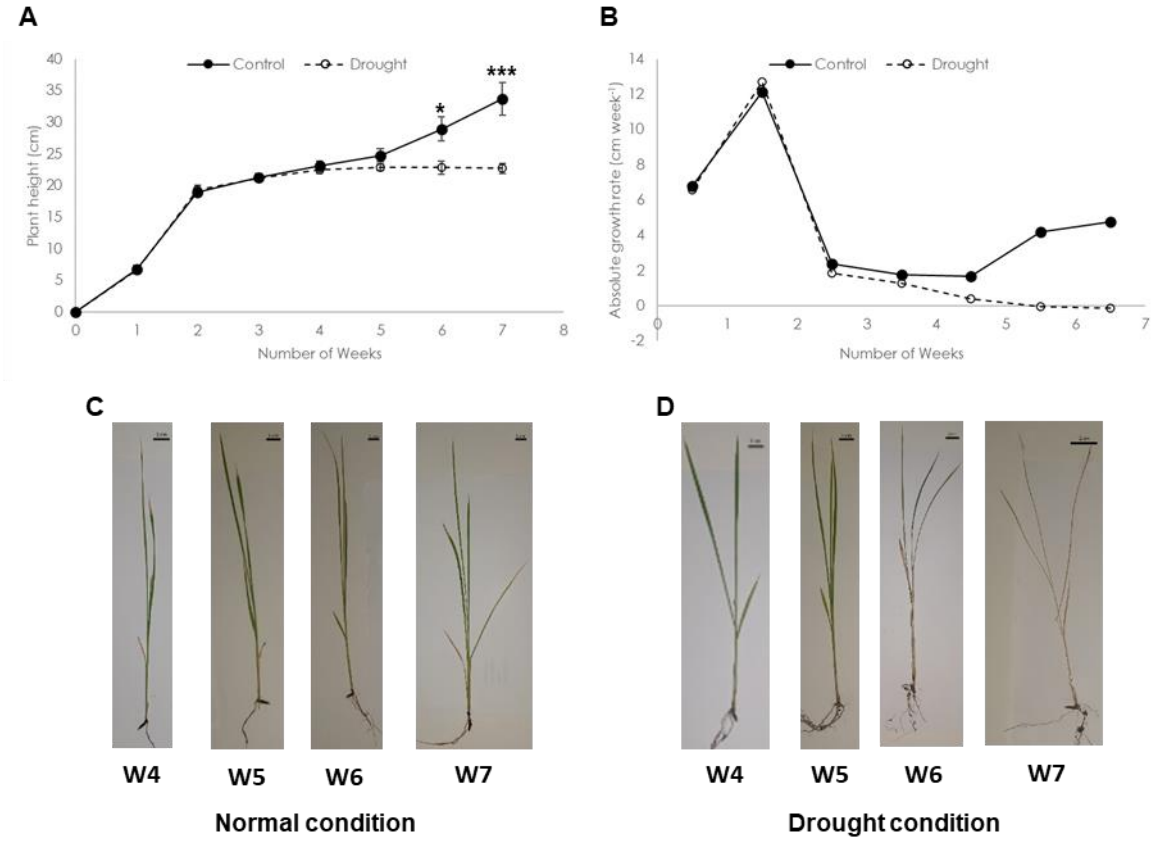

Figure 2. *Oryza sativa* ssp. *Indica* cv MR303 growth from week 0 until week 7. Drought treatment was started on week 3 onwards and drought-treated rice fully wilt on week 7. **(A)** MR303 growth curve and **(B)** absolute growth rate between rice grown in normal condition and under drought condition. Morphology of rice plantlet were also recorded after first week of drought treatment (W4), until its fully wilt in **(C)** control and **(D)** drought-treated condition. Statistical t-test: *P≤ 0.05, ***P≤ 0.001

RNA quality and semi-quantitative RT-PCR

Validation of extracted RNA integrity and purity were determined by agarose gel visualization and ratio of absorbance A_{260}/A_{280}. Gel electrophoresis showed an intact band with the presence of 18S and 28S rRNA indicates the good integrity of the RNA (Figure 3). The purity of the RNA extracted from control and drought-treated leaf were also in acceptable range based on the absorbance reading which is 2.03 and 1.98 respectively. As for the concentration, control plant RNA yield is 619.78 ng/µl whereas drought-treated plant RNA yield is 1074.76 ng/µl. The total RNA was ensured to be free from DNA contamination by carrying out DNase treatment protocol.

For the semi-quantitative RT-PCR, cDNA generated from the extracted RNA were divided into three replicates for each condition. Three primers were used for this analysis which are *OsActin* as internal control, and our targeted genes *OsWRKY11* and *OsNAC2*. PCR products were then visualized in gel electrophoresis (Figure 3) for relative expression analysis.

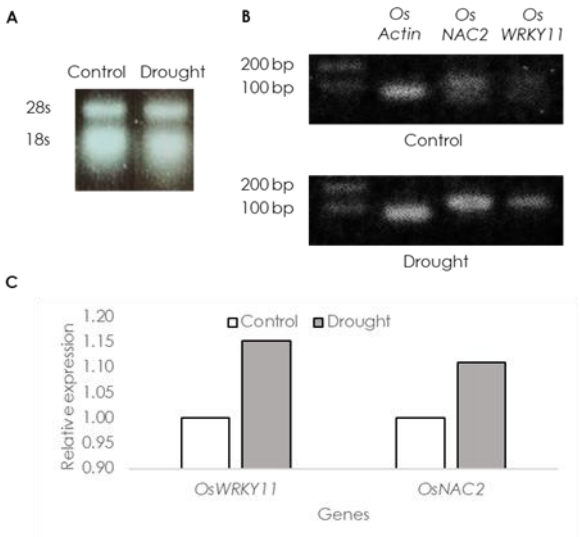

Figure 3. (A) Gel visualization of the extracted RNA from leaf of control and drought-treated plantlet. (B) PCR amplification of 3 genes: *OsActin*, *OsNAC2* and *OsWRKY11* for both control and drought-treated plantlet. 100 bp DNA ladder was used as reference. (C) Relative expression analysis of *OsWRKY11* and *OsNAC2* between control and drought-treated plant.

As shown in Figure 3, the band intensity of the *OsWRKY11* and *OsNAC2* cDNA were visually lower than the internal control. *OsActin*, a housekeeping gene which was stable throughout the different condition was used as an internal control [39]. Thus, we can negate the effect of drought condition to influence the expression of the *OsActin* as internal control which we used to quantify the relative expression of our targeted genes. Data normalization against the *OsActin* was conducted to remove internal variation influence for our data. Then, the relative expression was calculated using the ratio of targeted genes. From the results, drought condition causes the increment in *OsWRKY11* and *OsNAC2* gene expression. *OsWRKY11* has increased from 1 to 1.15 in drought-treated plantlet whereas *OsNAC2* has increased from 1 to 1.11 in drought-treated plantlet. There were 15% and 10% increment in relative gene expression of *OsWRKY11* and *OsNAC2* respectively. *WRKY* TF genes have been shown to positively expressed under abiotic stress [13]. Thus, we chose *OsWRKY11* as positive modulator marker for our study to get the expression overview on the drought-tolerance MR303 variety. Overexpression of the *OsWRKY11* has shown that it can prolong the duration of plant to survive under drought and increase it rate of survivability when water source become available again [28]. This gene expression was also increase in the MR303 variety which indicates that the *OsWRKY11* genes may contribute to the drought tolerance in this variety. For *OsNAC2*, we chose this gene as a negative modulator marker for our study. Previous study of *OsNAC2* in Nipponbare rice variety shows that rice plant become drought-sensitive when *OsNAC2* was overexpress while suppression of this gene using RNAi technology promotes drought-tolerance criteria in the rice plant [29]. These results contradict with our current findings in MR303 variety where the *OsNAC2* expression was increase when the plant was exposed to drought stress. Besides, previous findings using transgenic *Oryza sativa* L. spp *japonica* cv Nipponbare also shows that drought-tolerant trait enable the plant to survive up until 8 days when they overexpress *OsWRKY11* or 14 days when they suppress *OsNAC2* before it started to show wilting symptoms [28, 29]. However, our MR303 variety start to show wilting symptoms like browning and leaf shrinking after 3 weeks of drought treatment. Thus, these results suggest that our MR303 variety are more drought-tolerance and probably have different gene regulation to survive longer in the drought stress.

MR303 rice proline content under drought stress

Accumulation of proline in plants is an indication of disturbed physiological condition that triggered by biotic or abiotic stress condition. Free proline content can increase upon exposure of plants to drought, salinity, cold, heavy metals, or certain pathogens [36]. Determination of free proline levels is a useful assay to monitor physiological status and to assess stress tolerance of higher plants.

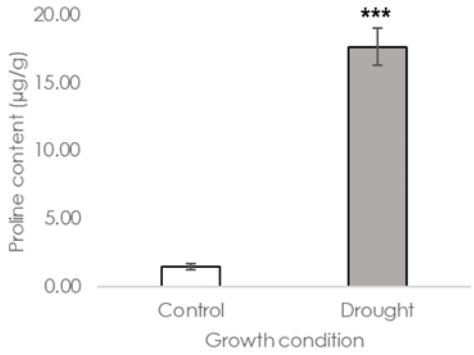

Figure 4. Proline content analysis on MR303 rice variety grown under normal (control) and drought conditions. Statistical t-test: ***P≤ 0.001.

From our analysis, there are significant increment in proline content for plantlet under drought condition (Figure 4). In plantlet under normal condition, the amount of proline is 1.48 ± 0.20 µg/g whereas, plantlet under drought condition have significantly higher proline content which is 17.64 ± 1.37 µg/g. There are more than 10 times increment of proline when the plantlet was exposed to drought stress. In response to drought stress, plants undergo osmotic adjustment in which proline plays an important role. Numerous studies have shown that proline not only acts as an osmotic protectant, but also as antioxidant [40]. Accumulated proline is thought to participate in stress-protective functions like antioxidants and stabilization of macromolecules during drought [41].

Future perspectives

This study has shown that MR303 variety drought-tolerance criteria may be contributed by the expression of *OsWRKY11* and *OsNAC2* TF genes under drought stress. This observation may be further elaborate through functional studies specifically in our local varieties. Previous study on different rice variety, *Oryza sativa L.* spp *japonica* showed that *OsNAC2* act as negative modulator during drought stress. However, our local variety which mostly consist of *Oryza sativa L*. spp *indica* shows an opposite result of *OsNAC2* expression under drought stress. These may suggest that the regulation of *OsNAC2* is species-specific and may activate different mechanism of drought tolerance pathway which results in better drought-tolerance trait. Other than that, differentially expressed gene analysis may also contribute in understanding the synergistic effect of drought-tolerance related genes in MR303 rice variety and identify the important player that play major roles in surviving drought stress.

Conclusions

In this study, we have confirmed that *OsWRKY11* and *OsNAC2* are positively regulated under drought stress. Higher relative expression of both genes was observed using semi-quantitative RT-PCR when comparing MR303 rice variety that grow under normal condition and under drought stress. Proline content in drought-treated plant were 10 times higher compared to the normal plant. Further studies may be conducted to fully elucidate the relation and roles of these genes in increasing drought-tolerance trait in our local rice variety.

Conflicts of Interest

The authors declares that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

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