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Unfolded protein response in rice (*Oryza sativa* L.) varieties with different level of salt stress tolerance

Galang Rizki Ramadhan[1,4], Sholeh Avivi[2,4], Bambang Sugiharto[3,4], and Wahyu Indra Duwi Fanata[2,4,*]

[1]Department of Biotechnology Graduate School Program University of Jember, Jl. Kalimantan Tegalboto, Jember, Jawa Timur 68121, Indonesia

[2]Department of Agrotechnology Faculty of Agriculture University of Jember, Jl. Kalimantan Tegalboto, Jember, Jawa Timur 68121, Indonesia

[3]Department of Biology Faculty of Mathematic and Natural Science University of Jember, Jl. Kalimantan Tegalboto, Jember, Jawa Timur 68121, Indonesia

[4]Center for Development of Advanced Science and Technology (CDAST) University of Jember, Jl. Kalimantan Tegalboto, Jember, Jawa Timur 68121, Indonesia

*Corresponding author: wahyuindra.faperta@unej.ac.id

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ABSTRACT Plants activate the unfolded protein response as part of cellular adaptation, thereby maintaining the endoplasmic reticulum homeostasis during external stresses exposure. In this study, we examined the relationship between the degree of salt tolerance and unfolded protein response-related gene expression in India salt-tolerant Pokkali and INPARI 35 varieties compared to the Indica salt-sensitive counterpart IR64 and INPARI 4 varieties. Our result showed that the salt tolerance of Pokkali and INPARI 35 had been confirmed by their higher survival rate, higher chlorophyll content, lower electrolyte leakage, and lower H2O2 and malondialdehyde content under salt stress conditions. Furthermore, the expression of unfolded protein response genes was highest in INPARI 35, whereas IR64 and INPARI 4 exhibited low gene induction during endoplasmic reticulum stress conditions. Among the four examined varieties the salt tolerant Pokkali surprisingly showed the lowest induction of all examined unfolded protein response-related genes. These results indicated the possibility that unfolded protein response supports the rice plant for adapting to the saline environment.

KEYWORDS Rice; salinity stress; endoplasmic reticulum stress; unfolded protein response; gene expression

1. Introduction

Soil salinity has become one of the major constraints that negatively affect rice productivity worldwide. As the glycophyte cereal plant, rice generally exhibits low adaptability against high concentrations of NaCl that present in the rhizosphere area. Salinity stress occurs when the concentration of sodium (Na^+) and chloride (Cl^-) ions in the soil solution exceeds the tolerable limit of rice. Salt stress affects the physiology and biochemistry processes of rice by given water stress, disturbs ion homeostasis, nutritional disorders, alteration of metabolic processes, oxidative stress, reduction of growth and cell division, and membrane disorganization (Ya'acob et al. 2017).

Rice that are grown under high salinity conditions experience both osmotic and ionic stresses. Osmotic stress occurs when the concentration of Na+ is high in the roots. High concentration NaCl in the soil solution leads to the decreased concentration of potassium (K^+), displacement by Na^+, depolarization of cell membrane. The decrease of K+ also caused decreased turgor cell and stomata closure (Ragel et al. 2019). Rice has several mechanisms to respond the stress including, the accumulation of osmoprotectant compounds, ion homeostasis, reactive oxygen species (ROS) detoxification, and activated several genes to maintain the balance of physiological processes of the cells (Hoang et al. 2016). A previous study reported an increased ROS production in rice plants was mediated by a specific gene in response to high extracellular NaCl concentration (Laohavisit et al. 2013).

Endoplasmic reticulum (ER) is responsible for synthesizing half of eukaryotes protein, but its function is easily disrupted when the cells are exposed to unfavorable environments. The disturbed ER function leads to the accumulation of misfolded and unfolded protein in the lumen, which subsequently generates the condition commonly termed as ER stress. In order to re-establish ER homeostasis, plant activated unfolded protein response (UPR) by increase protein folding capacity through the up regulation of a subset genes encoding the ER-localized chaper-

one protein and degrading misfolded proteins by triggering the ER-associated protein degradation (ERAD) mechanism (Liu and Howell 2016; Fanata et al. 2013). In rice, *OsbZIP39* and *OsbZIP74* transcription factors play a pivotal role in UPR activation. ER stress activates both transcription factors through ER to nucleus relocation by proteolytical cleavage of *OsbZIP39* and *OsbZIP74* mRNA unconventional splicing resulting in up-regulation of several ER chaperones such as *BiP1, PDIL1-1, Calnexin*, and *Calreticulin* (Lu et al. 2012; Takahashi et al. 2012).

Our experiment used two salt tolerant rice varieties, Pokkali and INPARI 35, altogether with IR64 and INPARI 4 as sensitive varieties to investigate the relationship between salinity stress tolerance and UPR induction level. The salt tolerance level and expression of ER chaperone genes in each variety have been analyzed through phenotypic, biochemical, and molecular methods.

2. Materials and Methods

2.1. Plant material and growing condition

The seedling of INPARI 35, Pokkali, INPARI 4, and IR64 rice were used in this study. Pokkali and INPARI 35 are classified as potential rice tolerance varieties, while INPARI 4 and IR64 are salt stress susceptible varieties (Kumari et al. 2018; **?**). Rice seeds were imbibed in the water for three days and twenty germinated rice seeds of each variety were planted in pots containing natural soil. The plants were grown for two weeks in a greenhouse with 28–32 °C temperature under natural sunlight exposure and daily irrigated by submerging the three-fourths of the pots into the water.

2.2. Salt Tolerance Analysis

Salt tolerance analysis was conducted by soaking the pots of 2-week-old rice seedling into 100 mM NaCl for six days. The salt tolerance of each rice variety was manifested by the survival rate at the six days of salt stress treatment by counting the percentage of living plants over the total population of the seedling.

2.3. Relative Electrolyte Leakage

Electrolyte leakage ratio was measured according to Ueda et al. (2013). The leaves of salt stress- and non-treated seedling were cut into 0.5–1 cm length and 0.5 g of small cute leaves were gently shaken in 20 mL distilled water in flat bottomed Makarthy tube for 24 h at room temperature. Electrolyte conductivity of the water was measured using a conductivity meter (Horiba Scientific) and the result was expressed as EC1. The tubes containing mixture water and leaves were autoclaved at 120 °C for 15 min, and the electrolyte conductivity of cooled water was measured to obtain the total electrolyte conductivity (EC2). Relative electrolyte leakage was counted using the formula: EC1/EC2 × 100%.

2.4. Total Chlorophyll

Total chlorophyll was measured according to the method of Arnon (2018); Ma et al. (2018) with modification. Two hundred milligram of small-cut fresh leaves were placed into 15 mL conical tube containing 10 mL absolute ethanol. The mixtures were gently agitated for 48 h at room temperature. Chlorophyll content was analyzed by measuring the OD value of ethanol solution using Hitachi U-2900 spectrophotometer at 649 nm and 665 nm. Total chlorophyll was expressed as mg g^{-1} fresh weight.

2.5. Hydrogen peroxide content

Hidrogen peroxide content of salt stress treated rice was measured according to the method described by Velikova et al. (2000). Three hundred milligram of fresh leaves were ground and homogenized with 3 mL of 0.1% (v/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the resulting supernatant was transferred into a new microtube. The reaction mixtures were subsequently made by mixing 0.5 mL supernatant, 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. Absorbance was measured at 390 nm and the hydrogen peroxide content was expressed as µg g^{-1} fresh weight.

2.6. Malondialdehyde Content

Malondialdehyde (MDA) contents were measured according to the method described by Hodges et al. (1999). One hundred milligrams of fresh weight leaves from each stress treatment were ground and homogenized with 1 ml of 0.1% (v/v) trichloroacetic acid solution and centrifuged at 10,000 rpm for 5 min at 4 °C. Five hundred microliter of supernatant were mixed with 500 µL 0.5% (w/v) TBA in 20% TCA. The mixtures were reacted at 100 °C for 30 min and then stopped by incubating the mixture in ice for 10 min. Mixtures were subsequently centrifuged at 10.000 rpm for 10 min at 4 °C and the resulting supernatants were used for MDA measurement using spectrophotometry at 532 dan 600 nm. MDA content was calculated using an extinction coefficient 1.55 × 10^5 mM-1 cm-1 and expressed as µMg^{-1} fresh weight.

2.7. Total RNA Isolation and qPCR Analysis

Total RNA was extracted from the leaves of the control and 5 mM DTT treated seedling using RNAprep pure kit (Tiangen) following the manufacturer's instruction. One microgram of total RNA was used for cDNA synthesis using a Revertra Ace qPCR RT Kit (Toyobo). The qPCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad) and specific primer sets (Supplementary Table 1). *OsActin1* was used as internal reference gen to evaluate the transcriptional abundance of the selected UPR genes.

2.8. Statistical analysis

Each experiment of this study consisted of three biological replicates, and the statistical analysis was performed using

analysis of variance (ANOVA)..

3. Results and Discussion

3.1. The tolerance of rice against salt stress

To investigate the level of salinity tolerance of IR 64, INPARI 4, Pokkali, and INPARI 35 rice varieties, two-week-old seedlings were subjected to salt stress treatment by soaking the growing media with 100 mM NaCl. The tolerance level of each rice variety was represented as stress survival rate and total chlorophyll at six days of stress treatment. In our experimental condition, all seedlings of four rice varieties showed normal growth under non-stress conditions and growth reduction was obviously seen in NaCl treated seedlings (Figure 1a). Both Pokkali dan INPARI 35 showed the highest survival rate at 79.17% and 75.83%, respectively, in saline conditions. IR 64 and INPARI 4 showed lower survival rates at 51.67% and 47.50%, respectively (Figure 1b). The result above clearly showed that salinity tolerance of tolerant and sensitive varieties was significantly different.

The salt tolerance response of plants is usually shown by the higher chlorophyll content, which is related to the higher photosynthetic rate during salt stress (Solangi et al. 2016). To confirm the survival rate result, we analyzed the total chlorophyll content of seedlings under salinity and their membrane integrity using relative electrolyte leakage (REL) analysis. On the total chlorophyll content analysis, Pokkali and INPARI 35 varieties showed a slight decrease of chlorophyll content, whereas IR64 and INPARI 4 exhibited a dramatic decrease of total chlorophyll content compared to control (Figure 2). Furthermore, the REL analysis result showed that Pokkali and INPARI 35 exhibited a lower electrolyte leakage value of 30.72% and 27.18%, respectively, which statistically were not different from the control treatment (Figure 3). On the other hand, we observed the two-fold increase of electrolyte leakage in IR 64 and INPARI 4 at 70.25% and 66.33%, respectively, indicating NaCl treatment leading to severe membrane damage on both varieties. Therefore, these results confirm that seedlings of Pokkali and INPARI 35 showed higher vigor than IR 64 and INPARI 4 under salt stress exposure.

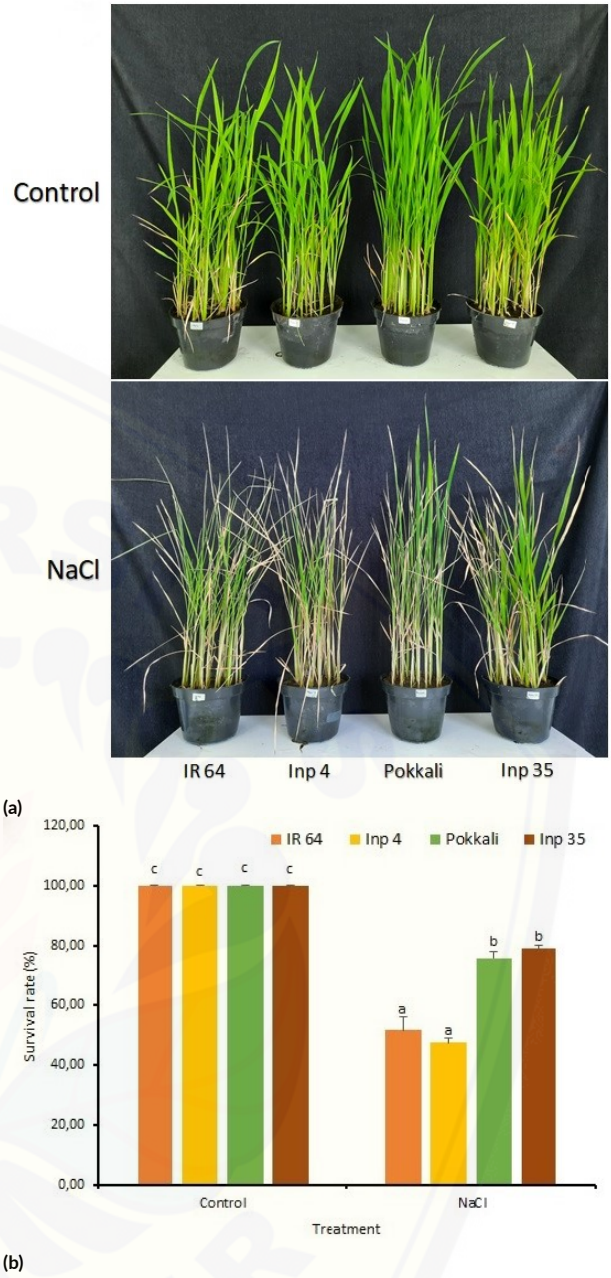

FIGURE 1 Effect of salinity stress on a) the phenotype and b) the survival rate of rice varieties IR64, INPARI 4, Pokkali, and INPARI 35 at 3 weeks seedling stage. Mean values of survival rate marked with the same letters do not differ significantly ($P \leq 0.05$) in LSD. The vertical bar showed the mean ± SE (n=3).

3.2. Oxidative stress level during salt stress

High accumulation of reactive oxygen species (ROS) during salt stress has induced oxidative damage of membrane lipids. To obtain the biochemical evidence for elucidating the different levels of salt tolerance on four tested rice varieties, we analyzed the oxidative stress level by measuring the H_2O_2 content and malondialdehyde (MDA) as the product of lipid peroxidation. Our data showed that salt stress has efficiently induced the production of H_2O_2 and MDA in all tested rice varieties and we noticed that the salt-sensitive IR 64 and INPARI 4 accumulate a higher level of and H_2O_2 and MDA than of Pokkali and INPARI 35 varieties. Compared to their respective control treatment, H_2O_2 accumulation in salt-treated IR 64 and INPARI 4 were increased by 29- and 39-fold, respectively, whereas Pokkali and INPARI 35 showed lower H_2O_2 induction levels (18- and 16-fold, respectively) (Figure 4). Moreover, the MDH content in IR 64 and INPARI 4 was increased by 60.8% and 33.6%, respectively, and Pokkali and INPARI 35 contained a lower induction level of MDH at 3.8% and 3.2%, respectively (Figure 5). These results were in accordance with the previous studies that rice tolerance varieties to salinity stress have the lowest amount of ROS than the sensitive plant (Hoang et al. 2015). Therefore, these results confirm that salt stress-induced oxida-

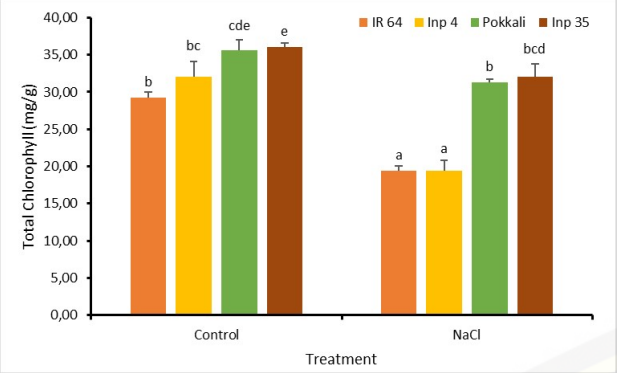

FIGURE 2 Effect salinity stress on the total chlorophyll rice varieties IR64, INPARI 4, Pokkali, and INPARI 35 at 3 weeks seedling stage. Mean values of total chlorophyll marked with the same letters do not differ significantly ($P \leq 0.05$) in LSD. The vertical bar showed the mean ± SE (n=3).

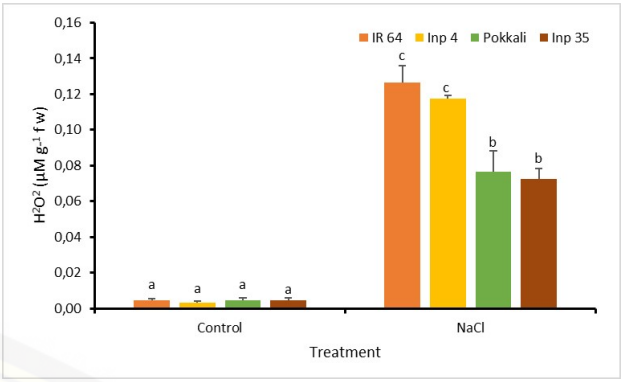

FIGURE 4 Effect salinity stress on the Hydrogen peroxide (H_2O_2) content in IR64, INPARI 4, Pokkali, and INPARI 35 at 3 weeks seedling stage. Mean values of H_2O_2 content marked with the same letters do not differ significantly ($P \leq 0.05$) in LSD. The vertical bar showed the mean ± SE (n=3).

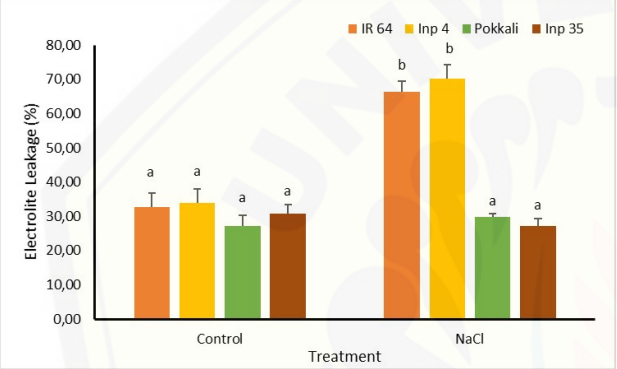

FIGURE 3 Effect salinity stress on the percentage of electrolyte leakage rice varieties IR64, INPARI 4, Pokkali, and INPARI 35 at 3 weeks seedling stage. Mean values of electrolyte leakage marked with the same letters do not differ significantly ($P \leq 0.05$) in LSD. The vertical bar showed the mean ± SE (n=4).

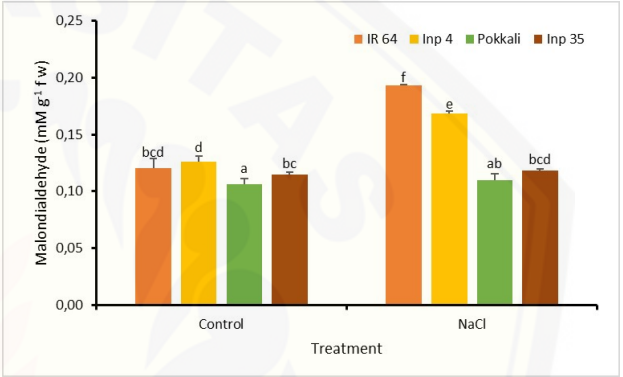

FIGURE 5 Effect salinity stress on the Malondialdehyde (MDA) in IR64, INPARI 4, Pokkali, and INPARI 35 at 3 weeks seedling stage. Mean values of MDA content marked with the same letters do not differ significantly ($P \leq 0.05$) in LSD. The vertical bar showed the mean ± SE (n=3).

tive stress was lower in salt-tolerant rice varieties but higher in the salt-sensitive counterparts.

3.3. Activation of unfolded protein response by ER stress

The phenotypical and biochemical results above have confirmed that Pokkali and INPARI 35 are categorized as salt tolerance varieties, whereas IR 64 and INPARI 4 are salt sensitive. To investigate whether the difference of salt tolerance in the tested rice correlates with the induction level of UPR, we analyzed the expression level of several ER stress-inducible genes in rice seedlings that experience ER stress induced by dithiothreitol (DTT). The induction levels of genes encoding ER-resident chaperone such as; *binding immunoglobulin protein-1 (BiP1), binding immunoglobulin protein-2 (BiP2),calreticulin-2 (CRT2), protein disulfide isomerase-like 2-3 (PDIL2-3)*, and *Calnexin (CNX)* were analyzed by qPCR analysis. Our result showed that ER stress was efficiently induced by DTT, as shown by the up-regulation of all ER chaperone genes (Figure 6). Moreover, we found that the induction of ER chaperone genes was lower in salt-sensitive IR64 and INPARI 4, whereas the salt-tolerant INPARI 35 showed the highest in the induction of *BiP1*, *BiP2*, *PDIL2-3*, and *CNX*. Interestingly, we observed the lowest induction of *BiP2*, *CRT2*, *PDIL2-3*, and *CNX* during the ER stress in salt-tolerant Pokkali rice and the basal level of *BiP2* and *CRT2* in Pokkali rice were also lowest among the tested varieties. These results indicate that UPR induction level correlates with the salt tolerance of IR 64, INPARI 4, and INPARI 35 but the salt tolerance of Pokkali rice has a minor correlation with the UPR activation.

3.4. Discussion

To investigate the correlation between UPR and salt tolerance level, we used Pokkali and INPARI 35 rice to represent salt-tolerant varieties and IR 64 and INPARI 4 for the salt-sensitive counterparts. The salt tolerance level of these four varieties was confirmed by the higher survival rate of Pokkali and INPARI 35 and lower survival rate of IR 64 and INPARI 4. Pokkali and INPARI 35 rice seedlings also showed a higher chlorophyll content compare to IR 64 and INPARI 4 under salt stress conditions. Moreover, REL analysis also showed that Pokkali

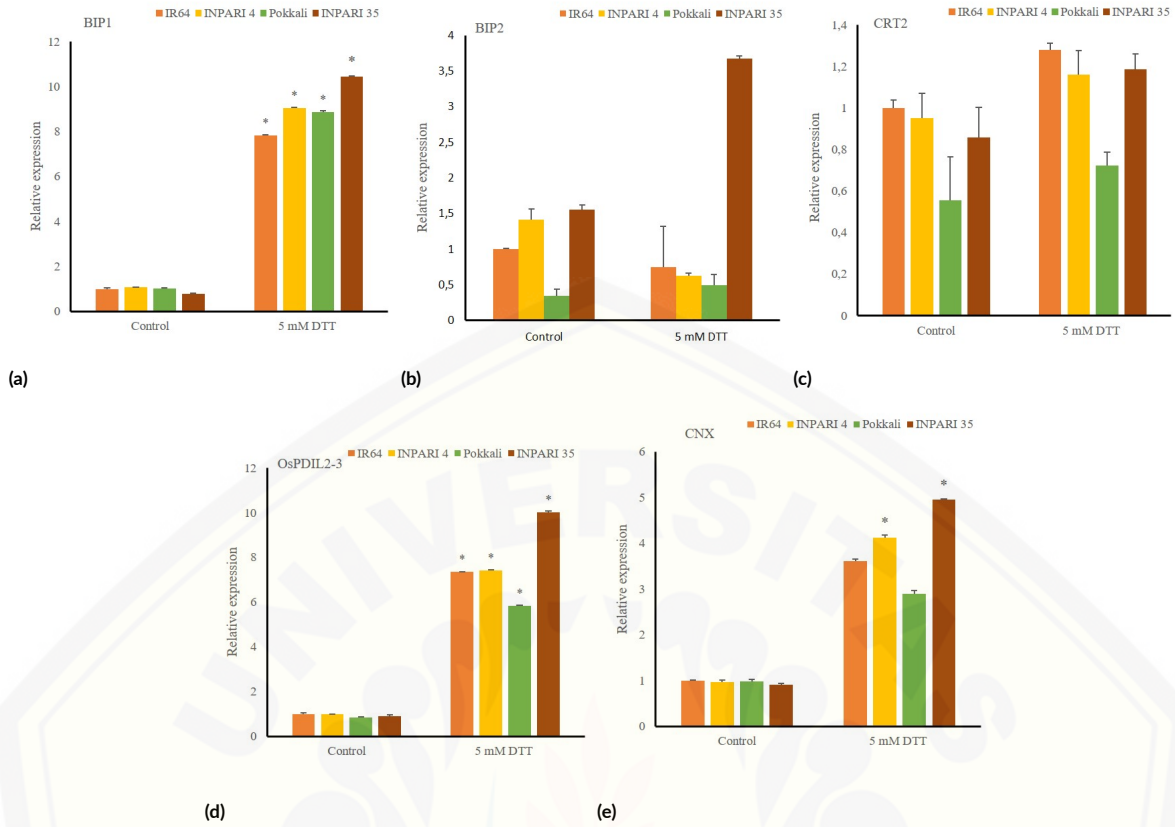

FIGURE 6 Relative expression of a) *BiP1*, b) *BiP2*, c) *CRT2*, d) *OsPDIL2-3* and e) *CNX* compared to that of *Actin1* detected by real-time qPCR. Values indicate relative expression levels in three biological replications from cDNA prepared from leaves. Vertical bars represent ± SE.

and INPARI 35 have a higher capability to maintain their membrane integrity during salt stress. The high salt accumulation in the cell induces chlorosis, leaf burning, and electrolyte leakage, which are the phenotypic symptoms in plants at the initial stage of salinity stress (Rahman et al. 2016; do Amaral et al. 2020). The salt-tolerant rice grown under high salinity exhibited less pronounced chlorosis and electrolyte leakage (Ma et al. 2018; Tang et al. 2019).

The cell that accumulated a high concentration of Na^+ generates more ROS that further contribute to the damage of cellular membrane (Ueda et al. 2013). Our result showed that H_2O_2 was highly accumulated in salt-sensitive IR 64 and INPARI 4 varieties, contributing to the higher lipid peroxidation activity as manifested by high MDA content in these salt sensitive varieties. These data were in accordance with the result of Abdelgawad et al. (2016) that showed the high level of H_2O_2, MDA and electrolyte in maize seedling leakage as the response of salt stress treatment.

The obvious salt tolerance level in the four tested rice varieties has become the main investigation object to correlate the salt tolerance level with the UPR. The salt-sensitive IR 64 and INPARI 4 showed the moderate activation of all tested genes under DTT-induced ER stress treatment. On the other hand, the salt-tolerant INPARI 35 showed the highest expression of *BiP1*, *BiP2*, *PDIL2-3*, and *CNX* among the tested varieties. The contrast results were shown by the salt-tolerant Pokkali where *BiP2*, *CRT2*, *PDIL2-3*, and *CNX* were expressed at the lowest level. BiP1 is known as the abundant ER chaperone protein and *OsPDIL2-3* also encodes a protein associated with protein folding and both genes were highly expressed under ER stress (Qian et al. 2015). It was also reported that rice overexpressed both *BiP1* and *CNX* under ER stress conditions, but CNX expression is inversely related to *BiP1* expression (Wakasa et al. 2011; Qian et al. 2015). Our result also showed that *CNX* was expressed inversely to that of *BiP1*. Interestingly, Pokkali rice showed the lowest expression of *CRT2* in both control and ER stress treatment. This result was in contrast to the positive function of calreticulin where the overexpression of wheat *CRT* in tobacco enhanced the drought and salt tolerance level (Xiang et al. 2015). Future investigation might be needed to deeply study the important roles *CRT2* for salt tolerance, especially in other salt-tolerant rice varieties.

4. Conclusions

Our phenotypical and biochemical results confirmed that Pokkali and INPARI 35 are salt tolerant, whereas IR 64 and INPARI 4 are salt sensitive. The activation of unfolded protein response through up-regulation of ER chaperone genes might support the rice plant adaptation to the

saline environment.

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Authors' contributions

GRR performed the experiment, collected the data, and analyzed data. SA, BS, WIDF designed the analysis, analyzed data. WIDF wrote the manuscript. All authors read and approved the final version of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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