

## Article

# Overexpression of Rice Metacaspase, *OsMC4*, Increases Endoplasmic Reticulum Stress Tolerance in Transgenic Rice Calli

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**Abstract:** Endoplasmic reticulum (ER) is an important organelle responsible as protein synthesis regulator in plant. High salinity can also lead to the activation of ER stress, caused by the accumulation of misfolded protein. This could lead to a stress response mechanism, unfolded protein response (UPR). Failure of UPR to reverse the effect of protein misfolding will activate Programmed Cell Death (PCD). Metacaspase genes regulate programmed cell death (PCD) in plants. The present study was focused on comprehensive gene analyses of the expression patterns of type II rice metacaspase (*OsMC*) genes in response to the endoplasmic reticulum (ER) and salinity stress in rice leaf and *OsMC4* in callus. A strong evidence of unfolded protein response (UPR) during tolerance to both ER and salinity stress was found in the present study. Overexpression of *OsMC4* in rice callus as a fusion protein with TagRFP and controlled by the CaMV35 promoter caused major changes in the expression of the stress ER-marker genes, protein disulfide isomerase (*PDI*) and Binding immunoglobulin Protein (*BiP*), and *OsMC4* in overexpressing calli. These expression analyses of the *OsMC* family provide valuable information for further functional studies on the biological roles of *OsMCs* in PCD related to ER and salinity stress responses.

**Keywords:** Transgenic rice, metacaspase, ER stress, salinity stress, programmed cell death

## 1. Introduction

Endoplasmic reticulum (ER) is a factory for protein synthesis in the cells. Folding of nascent peptides, packaging and delivering them to the targeted placement in the body are part of the ways in which this large organelle controls the protein homeostasis network [1,2]. When the capacity in the ER is overloaded, and the demand for protein folding increases, there will be disruption in the ER homeostasis [2]. This will cause excessive accumulation of unfolded protein in the ER which leads a situation referred as ER stress [3]. There are various stress responses that could occur together and linked to the ER [4].

One of the tolerance mechanisms in plants during ER stress is unfolded protein response (UPR), which is activated upon the accumulation of misfolded proteins in the ER [5]. Excessive accumulation of misfolded proteins can trigger a stress response

mechanism in plants called unfolded protein response (UPR). Various genes are involved in the UPR signaling pathway. For example, two transcription factors of the 'basic leucine ZIPper' (*bZIP*) family, *bZIP28* and *bZIP17*, which are type II transmembrane proteins. These proteins can only stay in the ER when they are attached to Binding immunoglobulin Protein (*BiP*) on its C-terminal tail. When plants are subjected to ER stress, *BiP* detaches itself from *bZIP28/17*, releasing *bZIP28/17* from cytosol to the nucleus and activates UPR-targeted genes, to help plant cells to refold the accumulating misfolded proteins and maintain homeostasis in plants. The same thing occurs in protein kinase, inositol-requiring enzyme 1 (IRE1) and its target *bZIP60*. They are inactivated when *BiP* is attached to them. IRE1 can only be activated when there is *BiP* disassociation, translocating the *bZIP60* transcription factor from cytosol to nucleus to initiate the upregulation of the UPR-targeted gene [6,7]. All the genes mentioned are directly involved in the UPR pathway in response to protein misfolding during ER stress.

In response to multiple biotic and abiotic factors, plant cell will respond with a series of the transcription and translation program in the ER stress response regulators. Abiotic stresses, such as drought and high salinity can also lead to the activation of ER stress [7,8]. For instance, in *Arabidopsis thaliana*, the salt stress signaling pathway is reported to resemble plant response towards ER stress [9]. Similar to what happens in the UPR pathway in response to ER stress, the salt treatment in *Arabidopsis* showed activation of the Golgi apparatus-resident site-1 protease (S1P) which then cleaves ER membrane-associated transcription factor *bZIP17*. Another type of enzyme involved in UPR as chaperone is protein disulfide isomerase (*PDI*), an enzyme responsible for catalyzing the disulfide bonds formation or breakage during protein folding [10].

If UPR is unable to control and reduce the effect of ER stress in plants, the cell will activate their apoptotic program called Programmed Cell Death (PCD) [11]. All living organisms possess a very crucial cell suicide system in order to maintain an appropriate metabolism function [12]. This system is commonly known as programmed cell death or PCD. It is widely known as a defense mechanism in organisms, usually activated in response to various biotic and abiotic stresses such as pathogens or through various other signaling molecules. This defense mechanism is of very significant importance for the survival of all multicellular organisms [13], particularly for growth, immune systems, tissue homeostasis and cell differentiation [14]. One type of PCD that is most studied and properly characterized is called apoptosis, which occurs in the animal kingdom. In spite of major hallmarks of apoptosis being observed in many plant PCD systems, many major regulators of apoptosis found in animals are absent in the plant genomes [15]. However, the terms PCD and apoptosis are often used together since apoptosis commonly occurs in cells dying under physiological conditions and often seen "programmed" since gene products will come together to conciliate the process [16]. Developmental PCD is vital in plants for the reproduction, growth of tissues and organ functions and also for efficient nutrient uptake [17]. PCD can be induced after an exposure to various stimuli including endoplasmic reticulum (ER) stress.

When in stressful condition, PCD will occur as a defense mechanism and cell will die and start losing cell membrane integrity, releasing electrolytes such as K<sup>+</sup> ions, in response to the stress. Consequently, the amount of electrolytes released from a tissue would correspond the cell death happening in the tissue. In order to quantify this leakage, the increase in electrolytic conductivity of water after affected tissue is immersed in it for an amount of time. This assay has been applied to measure and quantify the relative quantity of dead cells after exposure to abiotic and biotic stresses [18].

The route to PCD was assumed to be conserved throughout all kingdoms until the complete sequence of *Arabidopsis thaliana* was successfully obtained revealing that there was no presence of caspases in plant genomes. The regulators of PCD, metacaspases, a caspase-like protein, were then discovered in plants [19]. How PCD works in plants remained a mystery until the role of metacaspase in developmental PCD

in plant first demonstrated in Norway spruce, *Picea abies*. The metacaspase gene of *Picea abies*, mCI-Pa was found to have a very crucial function in making sure that cell degeneration, cell proliferation and terminal differentiation during embryogenesis are properly balanced [20].

Genome-wide characterizations in plant species revealed that metacaspases families are made up of multiple genes in plants. For instance, *Arabidopsis* has nine metacaspases [21,22], while eight members were found in potato (*Solanum tuberosum* L.) [23]. Through various molecular and phylogenetic studies, two different types of plant metacaspases, type I and type II, differentiated by their protein structures have been identified. Type I metacaspase is characterized by an N-terminal extension prodomain from 80-120 amino acids in length and two CxxC-type zinc finger domains. It is easy to differentiate between type I and type II metacaspases since the prodomain and domain are totally absent in type II metacaspases [22]. Both types, however, possess a p20 and p10 subunits in their C-terminal regions. These regions can still be differentiated between the two since the subunits in type I is slightly shorter than the one in type II [24]. In order to understand the critical role of metacaspases in plants, and how they involve in growth and stress responses, various gene functional studies were done by gene overexpression or silencing. For example, when Metacaspase 9 (*Camc9-C*) in pepper (*Capsicum annuum*) was silenced, it was found that the cell death was 30% higher in control plants as compared to the plant with silenced *Camc9-C* [25]. Meanwhile, when Metacaspase 8 in *Arabidopsis thaliana* (*AtMC8*) was overexpressed, upregulation of PCD was observed in the cells after being induced with oxidative stress, while seeds and seedlings with knocked out *AtMC8* showed increased tolerance towards herbicide methyl viologen. This suggested that *AtMC8* was involved in PCD pathway when exposed with oxidative stress [26].

There are eight members of metacaspases in rice (*OsMC*) [24,27]. Type I metacaspases in rice are *OsMC1-3* and type II metacaspases are *OsMC4-8*. In this study, the focus will be given to *OsMC4* as one of type II metacaspase since type II metacaspases are similar to effector caspases in animals, which functions to execute PCD by cleaving target substrates within cells [24,28,29]. Previous studies in rice metacaspases were focused on abiotic stresses such as salinity and drought, and biotic stresses such as fungal infection. We performed gene expression analysis specifically on type II metacaspase gene in response to ER stress to study where it belongs in the UPR pathway and how rice respond to this stress. Gene expressions during plant response towards salinity stress were also analyzed since salinity was tightly related to inducing ER stress in plants [7].

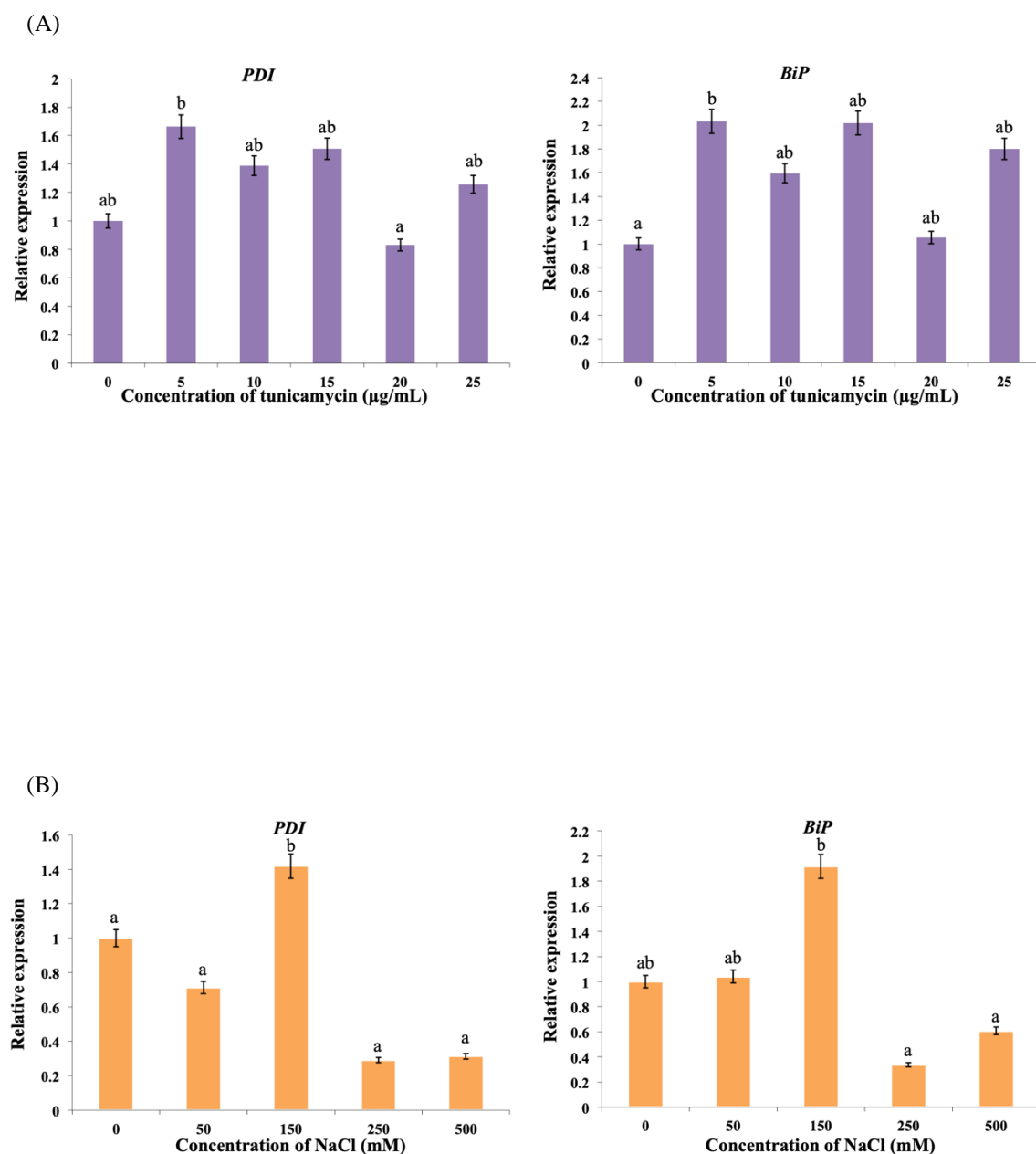
## 2. Results

### 2.1. Expression Patterns of ER-stress markers, *PDI* and *BiP*, in Response to ER and Salinity Stress under Various Concentrations

To analyse the gene expression analysis for metacaspases in rice and study its relation to ER and salinity stress tolerance in rice, *BiP* and *PDI* were used as stress markers in this study to confirm the ER stress-induced in rice. UPR can be induced in the lab by adding ER-stress inducers to plant tissues such as tunicamycin (TM), dithiothreitol (DTT) and cyclopiazonic acid (CPA) [30]. Salinity stress is induced by adding Sodium Chloride (NaCl) to rice tissues [31]. In this study, salt stress is used as a mean to induce ER stress and tunicamycin was used to induce ER stress in rice by inhibiting the N-glycosylation process and disrupting the post-translational modifications of glycoproteins [30].

qPCR analysis was first done for the expression of type II *OsMCs* in rice leaves after being subjected with ER stress for 3 hours under five different concentrations to test which concentration induces a strong and consistent ER stress in rice, hence should be used for further experiments. To characterize the intensity of the stress induced, two

stress markers, *BiP* and *PDI* were included in the analysis. As shown in Figure 1A, the expression of *BiP* and *PDI* in rice leaves treated with tunicamycin was significantly induced by up to two-fold with the 5  $\mu\text{g/mL}$  and 15  $\mu\text{g/mL}$  doses. Since salinity and drought stress were reported [7,8] to induce ER stress in rice plants, salinity stress was induced in rice leaves as a mean to induce ER-stress. ER-stress induction was also done directly via treatment with tunicamycin to study how these stresses correlate with each other. As shown in Figure 1B, the expression of *BiP* and *PDI* were most upregulated when rice leaves were induced with 150 mM NaCl for 3 hours. The upregulation of stress markers indicated that our trial tests were sufficient for further analyses. 5  $\mu\text{g/mL}$  of tunicamycin was observed to be enough to induce ER stress in leaves tissues and higher concentrations used did not show any significant difference to the amount of stress observed in rice leaves. Meanwhile, 150 mM NaCl was observed to bring significant changes to the stress level in rice leaves. Hence, rice leaves were further subjected to 5  $\mu\text{g/mL}$  tunicamycin and 150 mM NaCl under various time points to induce ER stress and salinity stress on rice leaves tissues, respectively.

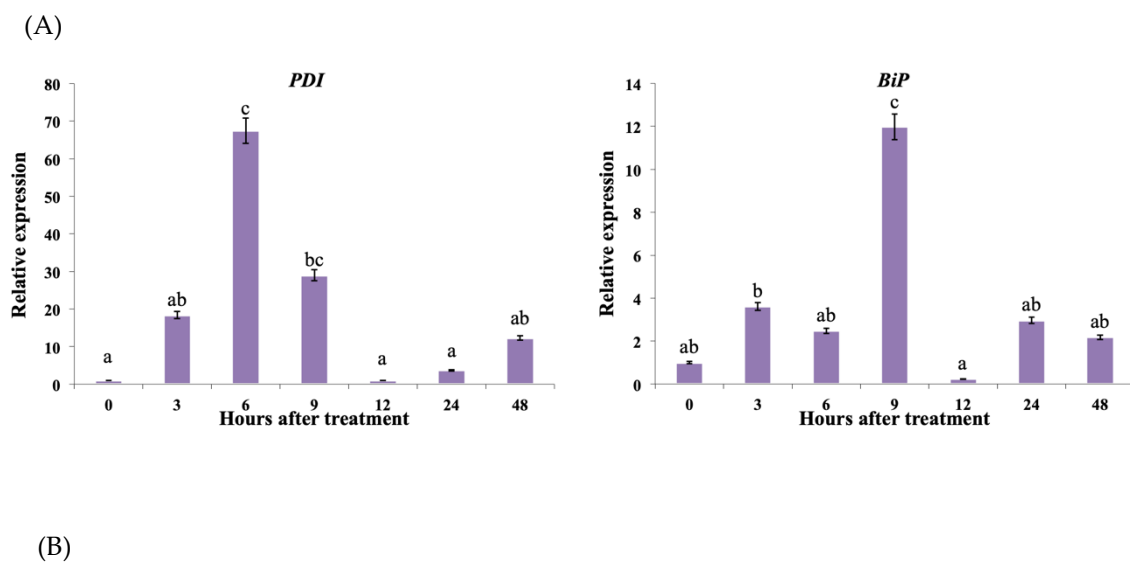


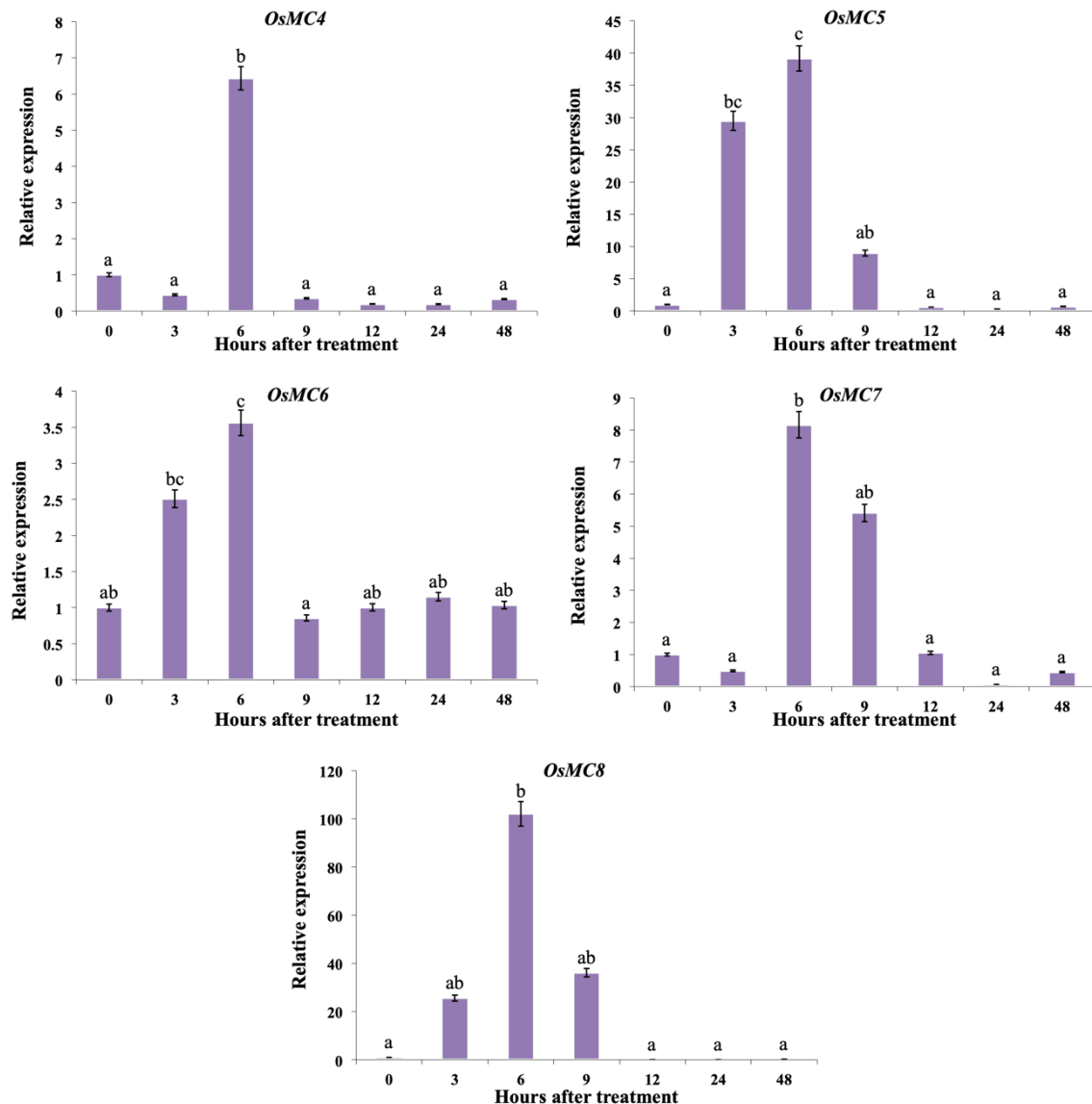
**Figure 1.** Expression of *PDI* and *BiP* in rice leaf after 3-hour ER stress treatment with various concentrations of (A) tunicamycin ( $\mu\text{g/mL}$ ) (B) NaCl. The expression patterns of *PDI* and *BiP* in both treatments were calculated with *UBQ5* reference genes and shown

as fold change of transcript level after setting untreated as 1. Data presented are the means  $\pm$  SD from three independent experiments and letters above the bars indicate significant difference at  $p < 0.05$  when analyzed using one-way ANOVA with Tukey's post-test.

## 2.2 Expression Patterns of ER-stress markers, *PDI* and *BiP*, and *OsMCs* in Response to ER Stress under Various Time Points

Expression patterns of type II *OsMCs* in the leaves under ER stress were analysed after being subjected to 5  $\mu\text{g/mL}$  tunicamycin for 3, 6, 9, 12, 24 and 48 hours. As shown in Figure 2B, a single peak pattern was seen on the transcript level of *OsMC4*, *OsMC5*, *OsMC6*, *OsMC7*, and *OsMC8* after 6 hours of the rice leaves being subjected to ER stress. The same patterns could also be observed with the transcript level pattern for *PDI* (Figure 2A). All the expression analyses peaked after 6 hours of treatment and then gradually decreased as shown in Figure 2A and 2B. *OsMC8* showed the most significant increase in expression after 6 hours of treatment (Figure 2B). About 100-fold change could be observed after 6 hours, then the expression gradually decreased to 20-fold change after 9 hours and significantly decreased after 12 to 48 hours of treatment. The expression pattern of *PDI* increased up to 60-fold change after 6 hours of treatment showing that the plant is substantially under a lot of stress (Figure 2A). Given the *PDI* expression pattern, a single peak for the expression of *BiP* was expected to occur at 6 hours of treatment, but instead was observed after 9 hours of ER stress (Figure 2B).



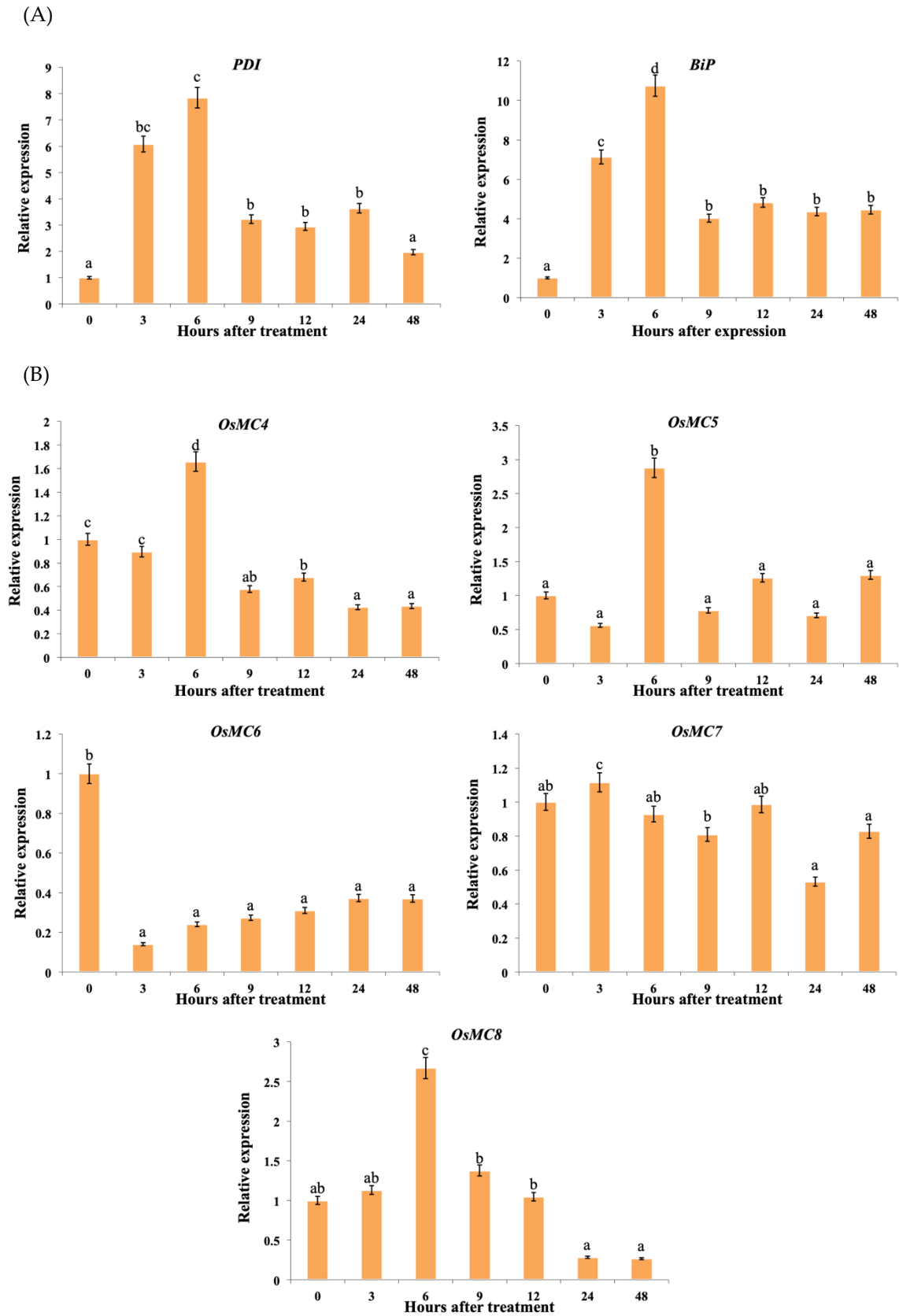


**Figure 2.** Expression of (A) ER stress markers *PDI*, *BiP* and (B) Type II *OsMCs* in rice leaf after treatment with 5 µg/mL of tunicamycin to induce ER stress at 6 time points. The transcript levels of *PDI*, *BiP* and *OsMCs* were all calculated using *UBQ5* as a reference gene and shown as fold change with the untreated sample set as 1. Data presented are the means  $\pm$  SD from three independent experiments and letters above the bars indicate significant difference at  $p < 0.05$  analyzed using one-way ANOVA with Tukey's post-test.

### 2.3 Expression Patterns of ER-stress markers, *PDI* and *BiP*, and *OsMCs* in Response to Salinity Stress under Various Time Points

Since salinity was reported to be able to induce ER stress [2], rice leaves were also subjected to 150 mM NaCl to induce salinity stress in rice and observe the relationship between salinity and ER stress. Rice leaves were layered with a thin coat of 150 mM NaCl for 3, 6, 9, 12, 24 and 48 hours. As shown in Figure 3A, the expression levels of *BiP* and *PDI* were observed to have a single peak after 6 hours of treatment and decreasing after 9 up to 48 hours of treatment. We can see up to 8-fold changes on *PDI* and up to 10-fold changes on *BiP*, 6 hours after treatment as compared to the corresponding control without stress treatment. As shown in Figure 3B, up to 2-fold changes were observed on the gene expression of *OsMC8* after 6 hours of treatment. The expression of *OsMC8* then gradually decreased up to 48 hours of treatment. Similar patterns could be observed in the expression of *OsMC4* and *OsMC5*, where a single peak could be seen on both genes after 6 hours of treatment before the gene expression started oscillating up to

48 hours of treatment. By contrast, fluctuations and irregular patterns were observed for *OsMC7*, while *OsMC6* is downregulated by the salt treatment.



**Figure 3** Expression of (A) ER stress markers *PDI*, *BiP* and (B) Type II *OsMCs* in rice leaf after treatment with 150 mM of NaCl to induce salinity stress at 6 time points. Expression patterns of *PDI*, *BiP* and *OsMCs* in this treatment were all calculated with *UBQ5*



reference genes and are shown as folds of transcript level. Data presented are the means  $\pm$  SD from three independent experiments and letters above the bars indicate significant difference at  $p < 0.05$  analysed using one-way ANOVA with Tukey's post-test.

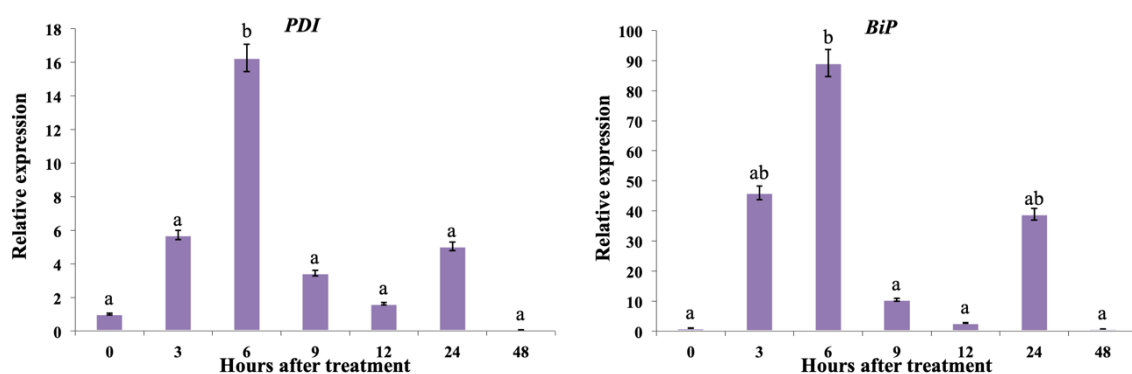
#### 2.4 Expression Patterns of ER-stress markers, *PDI* and *BiP*, and *OsMC4* on Wild Type and *OsMC4* Overexpressing Rice Calli in Response to ER Stress

Interesting patterns were observed on the expression pattern of *OsMC4*, *OsMC5* and *OsMC8*, warranting further investigation. In this study, we report on the overexpression of *OsMC4* in rice callus. The *OsMC4* gene construct was successfully amplified with the correct expected size after PCR, this gene was then used for cloning using the pGWB561 vector to express *OsMC4* as a fusion protein with TagRFP at the C terminus under the control of the CaMV 35 promoter.

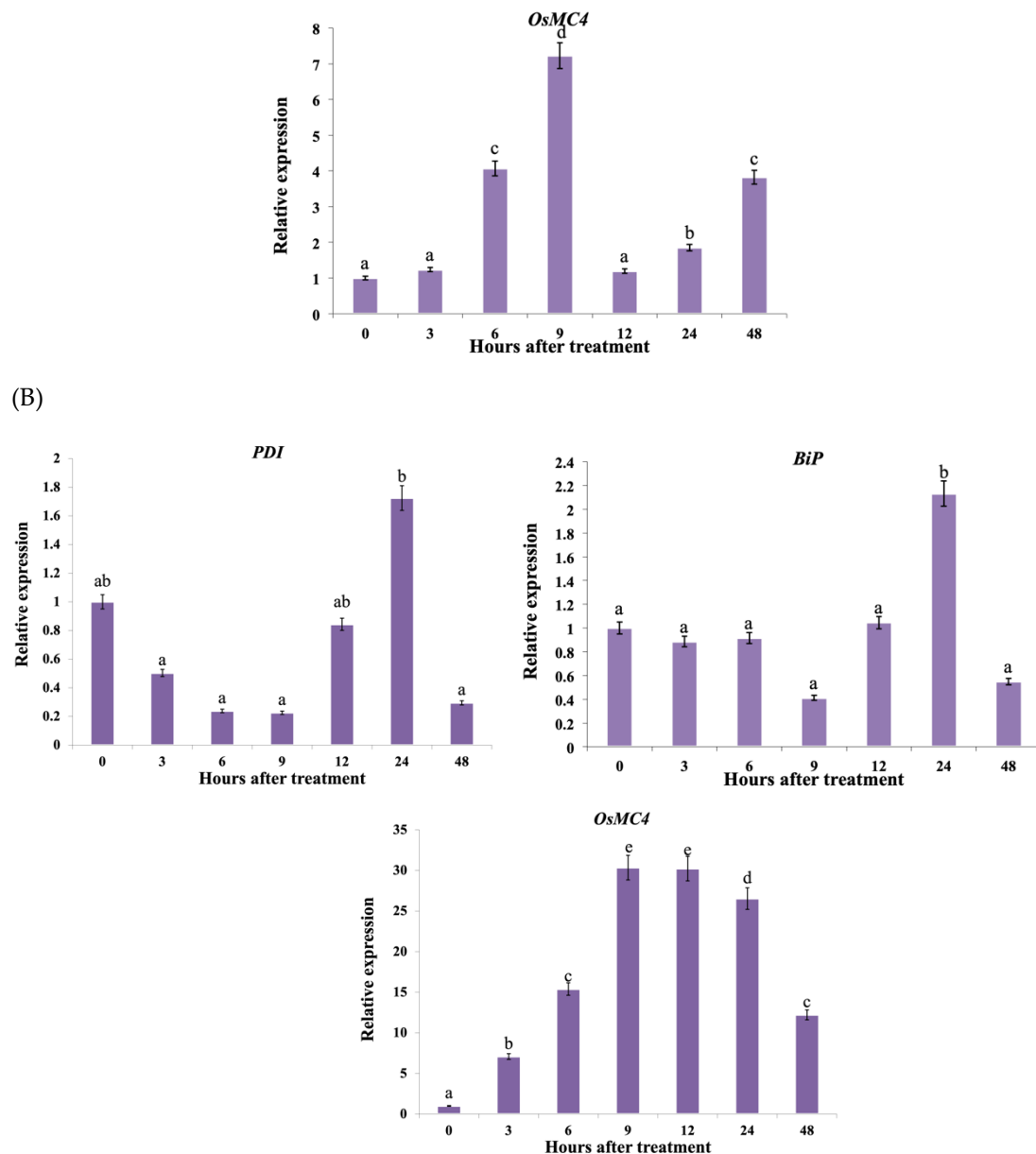
Approximately eight weeks after transformation in cultivated rice callus via *Agrobacterium tumefaciens*, and after being subcultured biweekly in selection media containing 250  $\mu\text{g/mL}$  cefotaxime, 250  $\mu\text{g/mL}$  carbenicillin and 50  $\mu\text{g/mL}$  hygromycin, positive transformants were recovered. Positive transformants were then treated with ER and salinity stress and subsequently being analysed for gene expression analysis. Transformation was confirmed using fluorescent testing with fluorescence microscope (ZEISS) since overexpressing calli comes with TagRFP fusion, giving them green fluorescence feature visible under fluorescence microscope.

Expression levels of *PDI*, *BiP* and *OsMC4* were measured and calculated using *UBQ5* as a reference gene. Data was obtained after expression and calculation using three biological replicates. As shown in Figure 4A and 4B, the expression levels of *BiP* and *PDI* were observed to decrease significantly from 16-fold and 90-fold highest expression for *PDI* and *BiP* in wild type, respectively, to only up to 2-fold changes in gene expression for both genes in overexpressing calli. However, a similar single peak pattern in the gene expression pattern of *PDI* and *BiP* observed in rice leaf could also be seen after 6 hours of treatment on the wild type rice calli. By contrast, the expression levels of *OsMC4* saw a huge difference between wild type and overexpressing calli, as expected (Figure 4A and 4B). The expression pattern fluctuates throughout the experiment but there was a significant difference in fold change between wild type and overexpressing calli with only 7-fold changes as the highest gene expression in wild type and 30-fold changes in overexpressing rice calli (Figure 4A and 4B). Comparing the results in wild type and overexpressing calli, the gene expression for stress marker genes, *PDI* and *BiP* was relatively high when *OsMC4* gene expression was low in wild type. Inversely, in overexpressing calli, *PDI* and *BiP* gene expression was observed to be low when *OsMC4* gene expression was high in overexpressing calli. Interestingly enough, the highest expression of *OsMC4* corresponded with the increase in expression of *PDI* and *BiP* from 9 to 24 hours after treatment in overexpressing calli. In wild type, the decrease in *PDI* and *BiP* gene expression also corresponded to the decrease in *OsMC4* gene expression from 6 to 12 hours after treatment.

(A)



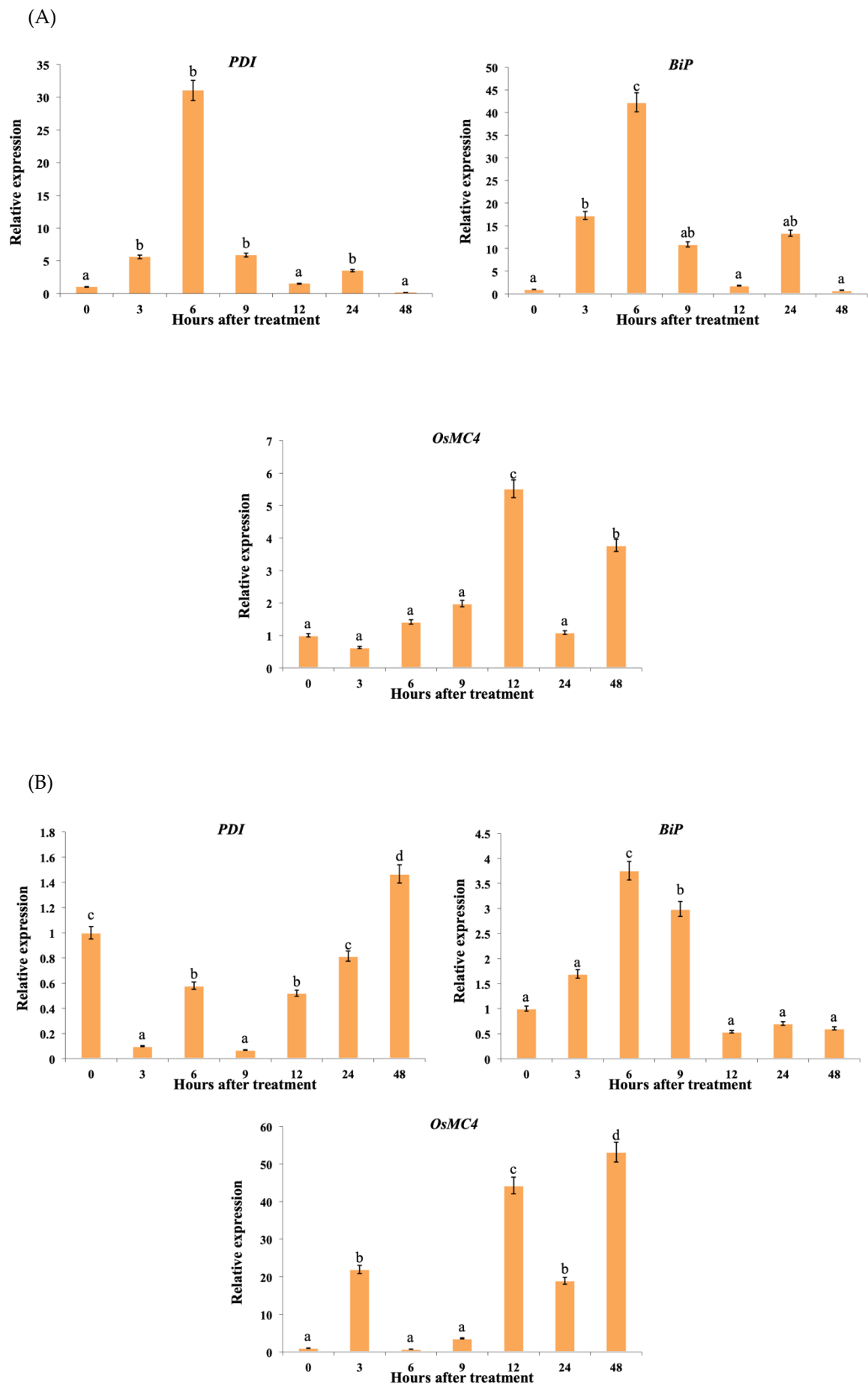




**Figure 4.** Expression of *PDI*, *BiP* and *OsMC4* in (A) wild type and (B) overexpressing calli treatment with 5 µg/mL tunicamycin to induce ER stress at 6 time points. Expression patterns of *PDI*, *BiP* and *OsMC4* in this treatment were all calculated using *UBQ5* as a reference gene and are shown as fold change compared to untreated. Data presented are the means ± SD from three independent experiments and letters above the bars indicate significant difference at  $p < 0.05$  analysed using one-way ANOVA with Tukey's post-test.

#### 2.5 Expression Patterns of ER-stress markers, *PDI* and *BiP*, and *OsMC4* on Wild Type and *OsMC4* Overexpressing Rice Calli in Response to Salinity Stress

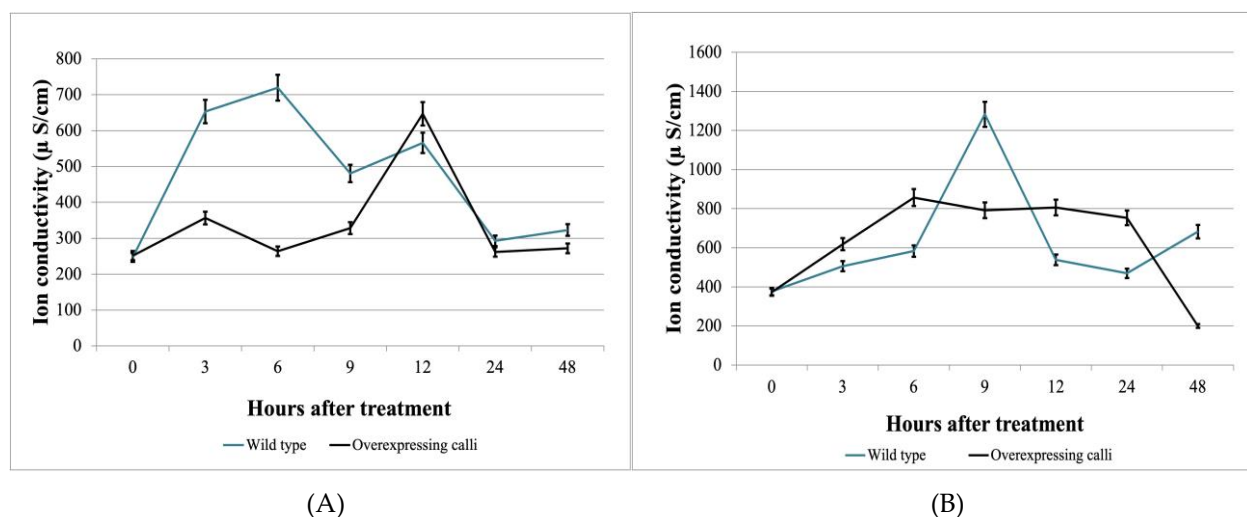
To explore if salinity stress would give the same effect towards gene expression on the wild type and the overexpressing calli, the same gene expression analysis was done for salinity stress after rice callus was subjected to 150 mM NaCl for 3 up to 48 hours of treatment. As shown in Figure 5A and 5B, the expression levels of *BiP* and *PDI* were observed to decrease significantly from 30-fold and 45-fold for *PDI* and *BiP* as the highest expression in wild type, respectively, to only lower than 2-fold changes in *PDI* and 4-fold in *BiP* in overexpressing calli. In contrast, the highest expression of *OsMC4* in wild type was only at 6-fold compared to 50-fold in overexpressing calli. The strong difference in the transcript level of the three gene expression could also be seen in ER stress in Figure 4A and 4B.



**Figure 5.** Expression of *PDI*, *BiP* and *OsMC4* in (A) wild type and (B) overexpressing rice callus after treatment with 150 mM NaCl to induce salinity stress under 6 various time points. Expression patterns of *PDI*, *BiP* and *OsMC4* in this treatment were all calculated with *UBQ5* reference genes and are shown as folds of transcript level. Data presented are the means  $\pm$  SD from three independent experiments and letters above the bars indicate significant difference at  $p < 0.05$  analysed using one-way ANOVA with Tukey's post-test.

### 2.6 Ion leakage analysis after the introduction of ER and salinity stress in rice

Ion leakage assay is a cell death assay done to further prove the condition of the cell during stress treatment. Under stressful condition, as a defence mechanism, cell will start dying and losing their cell membrane integrity, releasing electrolytes in response to the stress. Ion leakage assay is done to quantify the amount of electrolytes released during cell death [18]. As shown in Figure 6A and 6B, when applied with 5  $\mu$ g/mL tunicamycin to induce ER stress and 150 mM NaCl to induce salinity stress, the ion conductivity level was overall higher in wild type compared to overexpressing calli. The highest reading for ion conductivity post-ER stress as shown in Figure 6A was up to 700  $\mu$ S/cm in wild type and 600  $\mu$ S/cm in overexpressing calli. As for salinity stress in Figure 6B, the highest reading after plant was exposed to salinity stress was 1200  $\mu$ S/cm in wild type and 800  $\mu$ S/cm in overexpressing calli. This corresponds to the difference in *PDI* and *BiP* expression under both stresses in overexpressing calli and wild type.



**Figure 6.** Quantification of rice plant cell death by ion leakage assay. Reading of ion leakage ( $\mu$ S/cm) recorded after (A) ER stress and (B) Salinity stress were induced in wild type and overexpressing calli for 3, 6, 9, 12, 24 and 48 hours, respectively. Data presented are the means  $\pm$  SD from three independent experiments. Rice was treated with 5  $\mu$ g/mL tunicamycin to induce ER stress and 150 mM NaCl to induce salinity stress.

### 3. Discussion

The role of metacaspases in rice has been extensively discussed in a myriad of abiotic stress research [31]. However, the information of its role in ER stress in rice was limited as compared to other plants such as tomato [5], and Arabidopsis [32]. Role of metacaspases in salinity stress in rice is also quite new. Tunicamycin blocks glycosylation in the ER [30], while environmental stress like salinity stress could also lead to ER stress in plants [33].

The results observed in this study showed that type II metacaspases, especially *OsMC4*, might play a role in reducing ER and salinity stress in rice. Nonetheless, various responses were observed from each metacaspase. Some are more upregulated than the other after the stress was induced, and some are downregulated throughout the experiment. This showed that from all type II metacaspases, not all of them are directly

involved in regulating this type of stress. Different types of metacaspases might involve in different types of defence mechanisms. For example, *OsMC1* in rice was reported to be upregulated when rice samples were infected with blast fungus and high concentration of salicylic acid but were downregulated when pathogenic *Rhizoctonia solani* was introduced [31]. In this study, *OsMC8* was reported to be significantly upregulated after rice leaf was being subjected to ER stress (Figure 2B), but the gene expression was observed to be much lower after the rice leaves were introduced with salinity stress (Figure 3B). Therefore, the upregulation of *OsMC8* might imply its involvement in plant responses towards ER stress than salinity stress.

Two genes, *PDI* and *BiP* were used in this study as ER-stress marker genes. When plants are exposed to ER stress, *BiP* will be disassociated from ER stress sensors to initiate UPR pathway in response towards ER stress [6, 7], while *PDI* catalyzes disulfide bonds formation or breakage during protein folding to aid in protein folding in plants during exposure to stress [10]. In this study, the upregulation of UPR-targeted genes after 6 hours of treatment for most genes and 9 hours for *PDI* which resulted in a single peak on gene expression patterns were an indicator that UPR pathway was activated in rice. Rice activates its defence mechanism towards ER stress by initiating the UPR pathway [34]. This was supported by the high stress level shown by the stress marker genes *BiP* and *PDI* after 6 hours of ER and salinity treatment on rice leaves but gradually decreased right after (Figure 2B and 3B). ER stress and salinity stress can induce the upregulation of *BiP* transcription [35]. Certain rice metacaspases genes showed similar patterns after 6 hours of treatment. This is the case for *OsMC4*, *OsMC5*, *OsMC6*, *OsMC7* and *OsMC8* in rice leaves for ER stress (Figure 2B) but only *OsMC4*, *OsMC5* and *OsMC8* for salinity stress (Figure 3B). However, in the subsequent analysis using rice calli as tissue samples, for wild type and overexpressing calli, the peaks on 6 hours were mostly observed on the wild type, rather than the overexpressing calli (Figure 4 and 5). This shows how rice leaves and callus might have similar responses towards ER and salinity stress although they are both different types of tissues.

The peak for the ER-stress markers, *BiP* and *PDI* at the time points during gene expression may have been an indication that the rice leaves and callus were initially under a lot of stress, and that the stress increases in the first hours of the experiment. Subsequently, the expression gradually decreased for most of the metacaspases genes, suggesting that protein misfolding might have successfully be resolved by UPR-targeted genes (Figure 2B and 3B). However, the expression of *OsMC7* in salinity stress as shown Figure 3B, starts increasing again after the single peak pattern, and continued towards the end of the experiment. This might imply that this gene was dealing with a different stress in which the plants started releasing more *OsMC7* into the system. The sudden drop in the expression of most genes after the stress treatments on rice leaves as shown in Figure 2 and 3 (A and B) might also indicate that plants were either adapting or switching to PCD to stop the rescue mechanism, whereby cells started killing itself. This could be seen in ER stress in rice leaves, whereby the expression in *BiP* and *PDI* gradually increased after the single peak, showing the stress level was increasing, and more UPR-targeted genes were released, although the *OsMCs* for that treatment started to decrease after the peak (Figure 2A and 2B).

A much stronger UPR and a boost in the expression of *BiP* could be influenced by imperfection or deformities found in the UPR signalling pathway [30]. However, in this study, the overexpression of *OsMC4* has caused a much reduced expression of *BiP* and *PDI* genes, after treatment of overexpressing calli with ER. The callus with overexpressed *OsMC4* showed much higher expression of *OsMC4* gene in overexpressing calli. In wild type, the highest expression of *OsMC4* was only 7-fold but in overexpressing calli, the highest *OsMC4* expression was 30-fold (Figure 4A and 4B). Similarly, during salinity stress, the highest *OsMC4* expression was 6-fold in wild type and 50-fold in wild type (Figure 5A and 5B). The overexpression of *OsMC4* gene in rice plants might have helped increase their tolerance towards ER stress and salinity stress.

Overexpression of metacaspases genes in other plants has been demonstrated to

help relieve stress under extreme abiotic stress. For example, the overexpression of *AtMC8* in *Arabidopsis* has shown to be helpful in PCD induced by ultraviolet light and H<sub>2</sub>O<sub>2</sub> [26], while silencing of metacaspases gene could hinder plants from reacting properly towards stress. In this study, overexpression of *OsMC4* showed difference in the relative expression of *BiP* and *PDI* in both gene expression analysis and ion leakage assay. The ion conductivity reading in wild type is higher than in overexpressing calli. This also corresponds with the difference in *PDI* and *BiP* expressions under both stresses in overexpressing calli and wild type. Lower ion conductivity reading was observed for both *BiP* and *PDI* in overexpressing calli compared to wild type. Thus, in this study, overexpression of *OsMC4* has helped increase rice tolerance towards both ER and salinity stress.

#### 4. Materials and Methods

##### 4.1 Plants Materials and Growth

MR219 Rice (*Oryza sativa*) of indica rice hybrid by Malaysian Agricultural Research and Development Institute (MARDI) were used for gene expression analyses of Metacaspase genes on Endoplasmic Reticulum (ER) stress and salinity stress. The rice plants were grown under natural lighting and weather and were watered every 2-3 days for approximately 2-3 weeks.

##### 4.2 Treatments for Endoplasmic Reticulum Stress

Two-weeks old MR219 seedlings were submerged or layered with a thin coat of various concentrations of tunicamycin to test which concentration gives out the most stable and comparable gene expression in response to ER stress. Rice seedlings were subjected to five different concentrations of tunicamycin (5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL and 25 µg/mL) for 3 hours. Each tunicamycin was prepared with 0.02% Tween20 and diluted in DEPC-treated water (Invitrogen). After gene expression analyses, rice seedlings were subjected to ER stress using 5 µg/mL as the most suitable concentration for 6 different time points (3 hours, 6 hours, 9 hours, 12 hours, 24 hours and 48 hours). The same treatment was then done to induce ER stress to wild type and overexpressing calli. Callus was submerged or layered with a thin coat of 5 µg/mL of tunicamycin solutions prepared with 0.02% Tween 20.

##### 4.3 Treatment for Salinity Stress

The treatment was started with a treatment trial of 2-3 weeks old seedlings using 4 different concentrations of Sodium Chloride (NaCl); 50 mM, 150 mM, 250 mM and 500 mM diluted with DEPC water and 0.02% Tween20. After gene expression analyses, rice seedlings were treated with 150 mM NaCl as the most suitable concentration for 6 different time points (3 hours, 6 hours, 9 hours, 12 hours, 24 hours and 48 hours). The same treatment was then done to induce salinity stress to wild type and overexpressing. Callus was submerged or layered with a thin coat of 150 mM NaCl solutions prepared with 0.02% Tween 20.

##### 4.4 RNA extraction and cDNA conversion

All treated samples were collected and extracted using TRIsure™ (Bioline, UK). RNA purification was done using RNeasy plant mini kit (QIAGEN). All RNA samples were stored at -80 °C. The purified RNA was retro-transcribed into cDNA using the RT2 first strand kit (QIAGEN). cDNA samples were then ready for qPCR analysis.

##### 4.5 qRT-PCR Analysis for Gene Expression

qPCR analysis was done using SensiFAST SYBR Hi-Rox Kit (Bioline, UK) on a StepOne Plus™ machine from Applied Biosystems with a total volume of 10 µL. A total of 9 primers set were used for qPCR analysis post-treatment with ER and salinity stress. Two housekeeping genes; *UBQ5* (accession number AK061988) and *eEF1α* (accession

number AK061464) [36], two ER-stress marker; *BiP* (accession number AF006825) and *PDI* (accession number AB039278) [37], and 5 type II Metacaspases; *OsMC4*, *OsMC5*, *OsMC6*, *OsMC7* and *OsMC8* [31], genes were used for this analysis. Two housekeeping genes were used at first but focus was given to *UBQ5* for final analysis.

Table 1. Primers used in this study

Primers	Sequences (5'-3')	Size (bp)	References
UBQ5-F	ACCACTTCGACCGCCACTACT	69	Jain et al., 2006
UBQ5-R	ACGCCTAAGCCTGCTGGTT		
eEF-1 $\alpha$ -F	TTTCACTCTTGGTGTGAAGCAGAT	103	
eEF-1 $\alpha$ -R	GACTTCCTTCACGATTTTCATCGTAA		
PDI-F	CAGTCAAGGTGCCTTCCAGT	113	Cao et al., 2017
PDI-R	TTTGGTCAGGCTCAACGTGT		
BiP-F	CCGTGCCAGATTTGAGGAGT	130	
BiP-R	GGTGCTACCACCGACAAGAA		
OsMC4-F	TCGACGTTCGTGGAGATGCTC	126	
OsMC4-R	ATTCACGAGCCGCTGATCTT		
OsMC5-F	GTGCCAGACCGACCAGACAT	102	
OsMC5-R	CCGCTCTTCTCCGACAGGAT		
OsMC6-F	CCACACCGCAGGGTTCTTCAT	147	Huang et al., 2015
OsMC6-R	GTCCAGGCTGCTGAGTGTATCC		
OsMC7-F	ATACAGACCGTGCTGGCGTC	143	
OsMC7-R	AGGAATGGCGTCTCGGCGTT		
OsMC8-F	TCCGGCAAGTGCCTCGTAAC	150	
OsMC8-R	CAATGCGGTCGGTCACAGGAT		

4.6 Statistical analysis

The data were analysed by one-way ANOVA and Tukey’s tests ( $p < 0.05$ ) using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). qRT-PCR data were analysed using the 2- $\Delta\Delta C_t$  method.

4.7 Molecular Cloning and Overexpression of Metacaspase Gene

To over express *OsMC4*, gene-specific primer was used for the first PCR to amplify the gene using DNA as the starting material. The construct was then cloned into a pGEM vector and then sent for sequencing. Sequencing results showed that a correct gene construct was obtained, hence, Gateway Entry clone was made by adding attB1 and attB2 primer sequences to the extracted DNA by PCR. PCR amplification products were then ligated into special plasmids called Gateway donor vectors, Gateway™ pDONR™ 221 Vector (Invitrogen) by adding BP Clonase enzyme mix. Our destination



vector was pGWB561 (Nakagawa lab, Japan) with TaqRFP fusion. A cloning construct for *OsMC4* was successfully obtained and it was then cloned into the destination vector and transformed into callus via *Agrobacterium tumefaciens* strain EHA105.

#### 4.8 Plant Transformation

Callus cultivation was done in preparation for callus transformation. Firstly, seeds were peeled to expose the embryos and were plated on T1 media plates (Murashige & Skoog medium with B5 vitamins, L-Arginine, L-Glutamine & L-Asparagine). A several seed sterilization techniques were carried out to reduce the contamination rate. Firstly, seeds were left under running water for one hour before seeds were soaked and shaken in a solution containing fungicide and Tween-20 for 30 min. This step was repeated twice. After an hour, seeds were soaked and shaken in another solution containing Virkon and Tween-20 for 30 min. After that, seeds were transferred to a solution of pure Ethanol and treated for a minute. Lastly, seeds were introduced to another solution containing 70% Clorox and Tween-20 for another 30 min. Seeds were lastly rinsed 3 times with distilled water and left to dry up to 3 hours before being plated in the T1 media. Callus started to grow after 14-20 days. After the transformation of *OsMC4* into rice callus, callus was transferred into co-cultivation media for 3 days. Then, callus was transferred into selection media containing 250 µg/mL cefotaxime, 250 µg/mL carbenicillin and 50 µg/mL hygromycin for selection. The overexpressing calli was subcultured into fresh selection media every two weeks and positive transformants were selected after 2-3 months. Positive transformants were visibly lighter in shades since negative transformants die in selective media with antibiotics. Positive transformants were confirmed with fluorescent testing under fluorescence microscope (ZEISS).

#### 4.9 Ion Leakage

Ion leakage analysis was done as a cell death assay. Ion conductivity was measured after samples were treated with 5 µg/mL tunicamycin for ER stress or 150 mM NaCl for salinity stress for 3, 6, 9, 12, 24, 48 hours using compact conductivity meter LAQUAtwin-EC-11 (Horiba Ltd). 100 mg rice callus were submerged in 1 mL DEPC-treated water (Invitrogen) water in 24-well plate at room temperature for one hour prior to ion conductivity measurement after stress treatment. water for one hour. Three replicates were done for each selected time point. The conductivity meter was calibrated with standard KCl solution.

### 5. Conclusions

In order to increase our understanding of the functions of *OsMCs*, the present study was focused on the possible involvement of type II *OsMCs* especially *OsMC4* in ER stress and how salinity stress was related to ER stress through comprehensive analysis of the expression changes in rice leaves and rice callus treated with both stresses. Our data first displayed how rice metacaspases might be involved in the UPR pathway during plant response to stressful condition. Three metacaspase genes, *OsMC4*, *OsMC5* and *OsMC8* showed highest gene expression 6 hours after stress treatment in rice leaf before sudden drop in gene expression which might have been related to activation of plant rescue mechanism during protein misfolding. Meanwhile, *OsMC4*, might play a very important role in tolerance towards ER and salinity stress after its overexpression in this study helped to increase rice tolerance towards both stresses. Overexpression of this gene significantly reduced the level of *BiP* and *PDI* expression in rice, indicating that stress level is relatively lower in the successfully overexpressing calli. Results from the analysis of the *OsMCs* members provide clear evidence that metacaspase, particularly the *OsMC4* reduces ER and salinity stress in rice. Further functional analysis of *OsMCs* genes in ER and salinity stress tolerance should be done in the future to create higher stress tolerance in transgenic rice.

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## References

1. Chevet, E.; Cameron, P. H.; Pelletier, M. F.; Thomas, D. Y.; Bergeron, J. J. M. The endoplasmic reticulum: Integration of protein folding, quality control, signaling and degradation. *Current Opinion in Structural Biology* **2001**, *11*, 120–124. [https://doi.org/10.1016/S0959-440X\(00\)00168-8](https://doi.org/10.1016/S0959-440X(00)00168-8)
2. Ron, D.; Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology* **2007**, *8*, 519–529. <https://doi.org/10.1038/nrm2199>
3. Walter, P.; Ron, D. The unfolded protein response: From stress pathway to homeostatic regulation. *Science* **2011**, *334*, 1081–1086. <https://doi.org/10.1126/science.1209038>
4. Park, C. J.; Park, J. M. Endoplasmic reticulum plays a critical role in integrating signals generated by both biotic and abiotic stress in plants. *Frontiers in Plant Science* **2019**, *10*. <https://doi.org/10.3389/fpls.2019.00399>
5. Fu, C.; Liu, X. X.; Yang, W. W.; Zhao, C. M.; Liu, J. Enhanced salt tolerance in tomato plants constitutively expressing heat-shock protein in the endoplasmic reticulum. *Genetics and Molecular Research* **2016**, *15*. <https://doi.org/10.4238/gmr.15028301>
6. Pincus, D.; Chevalier, M. W.; Aragón, T.; van Anken, E.; Vidal, S. E.; El-Samad, H.; Walter, P. BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. *PLoS Biology* **2010**, *8*. <https://doi.org/10.1371/journal.pbio.1000415>
7. Tang, Y.; Bao, X.; Zhi, Y.; Wu, Q.; Guo, Y.; Yin, X.; Zeng, L.; Li, J.; Zhang, J.; He, W.; Liu, W.; Wang, Q.; Jia, C.; Li, Z.; Liu, K. Overexpression of a myb family gene, Osmby6, increases drought and salinity stress tolerance in transgenic rice. *Frontiers in Plant Science* **2019**, *10*, 1–12. <https://doi.org/10.3389/fpls.2019.00168>
8. Duan, Y.; Zhang, W.; Li, B.; Wang, Y.; Li, K.; Sodmergen, T.; Han, C.; Zhang, Y.; Li, X. An endoplasmic reticulum response pathway mediates programmed cell death of root tip induced by water stress in Arabidopsis. *New Phytologist* **2010**, *186*, 681–695. <https://doi.org/10.1111/j.1469-8137.2010.03207.x>
9. Liu, J. X.; Srivastava, R.; Che, P.; Howell, S. H. Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant Journal* **2007**, *51*, 897–909. <https://doi.org/10.1111/j.1365-313X.2007.03195.x>
10. Gupta, D.; Tuteja, N. Chaperones and foldases in endoplasmic reticulum stress signaling in plants. *Plant Signaling and Behavior* **2011**, *6*, 232–236. <https://doi.org/10.4161/psb.6.2.15490>
11. Chen, Y.; Brandizzi, F. AtIRE1A/AtIRE1B and AGB1 independently control two essential unfolded protein response pathways in Arabidopsis. *Plant Journal* **2012**, *69*, 266–277. <https://doi.org/10.1111/j.1365-313X.2011.04788.x>
12. Kroemer, G.; Martin, S. J. Caspase-independent cell death. *Nature Medicine* **2005**, *11*, 725–730. <https://doi.org/10.1038/nm1263>
13. Hofmann, K. The Evolutionary Origins of Programmed Cell Death Signaling. *Cold Spring Harbor Perspectives in Biology* **2020**, *12*. <https://doi.org/10.1101/cshperspect.a036442>
14. Van Hautegeem, T.; Waters, A. J.; Goodrich, J.; Nowack, M. K. Only in dying, life: Programmed cell death during plant development. *Trends in Plant Science* **2015**, *20*, 102–113. <https://doi.org/10.1016/j.tplants.2014.10.003>
15. Lam, E.; Zhang, Y. Regulating the reapers: Activating metacaspases for programmed cell death. *Trends in Plant Science* **2012**, *17*, 487–494. <https://doi.org/10.1016/j.tplants.2012.05.003>
16. Nagata, S. Apoptosis and Clearance of Apoptotic Cells. *Annual Review of Immunology* **2018**, *36*, 489–517. <https://doi.org/10.1146/annurev-immunol-042617-053010>
17. Daneva, A.; Gao, Z.; Van Durme, M.; Nowack, M. K. Functions and Regulation of Programmed Cell Death in Plant Development. *Annual Review of Cell and Developmental Biology* **2016**, *32*, 441–468. <https://doi.org/10.1146/annurev-cellbio-111315-124915>
18. Demidchik, V.; Straltsova, D.; Medvedev, S. S.; Pozhvanov, G. A.; Sokolik, A.; Yurin, V. Stress-induced electrolyte leakage: The role of K<sup>+</sup>-permeable channels and involvement in programmed cell death and metabolic adjustment. *Journal of Experimental Botany* **2014**, *65*, 1259–1270. <https://doi.org/10.1093/jxb/eru004>

19. Uren, A. G.; O'Rourke, K.; Aravind, L.; Pisabarro, M. T.; Seshagiri, S.; Koonin, E. V.; Dixit, V. M. Identification of paracaspases and metacaspases: Two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Molecular Cell* **2000**, *6*, 961–967. [https://doi.org/10.1016/s1097-2765\(05\)00086-9](https://doi.org/10.1016/s1097-2765(05)00086-9)
20. Suarez, M. F.; Filonova, L. H.; Smertenko, A.; Savenkov, E. I.; Clapham, D. H.; Von Arnold, S.; Zhivotovsky, B.; Bozhkov, P. V. Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Current Biology* **2004**, *14*, 339–340. <https://doi.org/10.1016/j.cub.2004.04.019>
21. Coll, N. S.; Vercammen, D.; Smidler, A.; Clover, C.; Van Breusegem, F.; Dangl, J. L.; Epple, P. Arabidopsis type I metacaspases control cell death. *Science* **2010**, *330*, 1393–1397. <https://doi.org/10.1126/science.1194980>
22. Tsiatsiani, L.; Van Breusegem, F.; Gallois, P.; Zavialov, A.; Lam, E.; Bozhkov, P. V. Metacaspases. *Cell Death and Differentiation* **2011**, *18*, 1279–1288. <https://doi.org/10.1038/cdd.2011.66>
23. Dubey, N.; Trivedi, M.; Varsani, S.; Vyas, V.; Farsodia, M.; Singh, S. K. Genome-wide characterization, molecular evolution and expression profiling of the metacaspases in potato (*Solanum tuberosum* L.). *Heliyon* **2019**, *5*, e01162. <https://doi.org/10.1016/j.heliyon.2019.e01162>
24. Wang, L.; Zhang, H. Genomewide survey and characterization of metacaspase gene family in rice (*Oryza sativa*). *Journal of Genetics* **2014**, *93*, 93–102. <https://doi.org/10.1007/s12041-014-0343-6>
25. Kim, S. M.; Bae, C.; Oh, S. K.; Choi, D. A pepper (*Capsicum annuum* L.) metacaspase 9 (Camc9) plays a role in pathogen-induced cell death in plants. *Molecular Plant Pathology* **2013**, *14*, 557–566. <https://doi.org/10.1111/mpp.12027>
26. He, R.; Drury, G. E.; Rotari, V. I.; Gordon, A.; Willer, M.; Farzaneh, T.; Woltering, E. J.; Gallois, P. Metacaspase-8 modulates programmed cell death induced by ultraviolet light and H<sub>2</sub>O<sub>2</sub> in Arabidopsis. *Journal of Biological Chemistry* **2008**, *283*, 774–783. <https://doi.org/10.1074/jbc.M704185200>
27. Liu, Q.; Xue, Q. Molecular phylogeny, evolution, and functional divergence of the LSD1-like gene family: Inference from the rice genome. *Journal of Molecular Evolution* **2007**, *64*, 354–363. <https://doi.org/10.1007/s00239-006-0131-x>
28. Bozhkov, P. V.; Filonova, L. H.; Suarez, M. F.; Helmerson, A.; Smertenko, A. P.; Zhivotovsky, B.; von Arnold, S. VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. *Cell Death and Differentiation* **2004**, *11*, 175–182. <https://doi.org/10.1038/sj.cdd.4401330>
29. Fortin, J.; Lam, E. Domain swap between two type-II metacaspases defines key elements for their biochemical properties. *Plant Journal* **2018**, *96*, 921–936. <https://doi.org/10.1111/tpj.14079>
30. Howell, Stephen H. Endoplasmic Reticulum Stress Responses in Plants. *Annual Review of Plant Biology* **2013** *64*: 477–99. <https://doi.org/10.1146/annurev-arplant-050312-120053>
31. Huang, L.; Zhang, H.; Hong, Y.; Liu, S.; Li, D.; Song, F. Stress-responsive expression, subcellular localization and protein–protein interactions of the rice metacaspase family. *International Journal of Molecular Sciences* **2015**, *16*, 16216–16241. <https://doi.org/10.3390/ijms160716216>
32. Cai, Y. M.; Yu, J.; Ge, Y.; Mironov, A.; Gallois, P. Two proteases with caspase-3-like activity, cathepsin B and proteasome, antagonistically control ER-stress-induced programmed cell death in Arabidopsis. *New Phytologist* **2018**, *218*, 1143–1155. <https://doi.org/10.1111/nph.14676>
33. Isayenkov, S. V.; Maathuis, F. J. M. Plant salinity stress: Many unanswered questions remain. *Frontiers in Plant Science* **2019**, *10*. <https://doi.org/10.3389/fpls.2019.00080>
34. Liu, Linchuan.; Li, J. Communications between the endoplasmic reticulum and other organelles during abiotic stress response in plants. *Frontiers in Plant Science* **2019**, *10*. <https://doi.org/10.3389/fpls.2019.00749>
35. Liu, Lijing.; Cui, F.; Li, Q.; Yin, B.; Zhang, H.; Lin, B.; Wu, Y.; Xia, R.; Tang, S.; Xie, Q. The endoplasmic reticulum-associated degradation is necessary for plant salt tolerance. *Cell Research* **2011**, *21*, 957–969. <https://doi.org/10.1038/cr.2010.181>
36. Jain, M.; Nijhawan, A.; Tyagi, A. K.; Khurana, J. P. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications* **2006**, *345*, 646–651. <https://doi.org/10.1016/j.bbrc.2006.04.140>
37. Cao, Z.; Zhao, Q.; Pan, G.; Wei, K.; Zhou, L.; Cheng, F. Comprehensive expression of various genes involved in storage protein synthesis in filling rice grain as affected by high temperature. *Plant Growth Regulation* **2017**, *81*, 477–488. <https://doi.org/10.1007/s10725-016-0225-4>