Phyton, International Journal of Experimental Botany
DOI: 10.32604/phyton.2020.08919
Article

Dithiothreitol and PEG Induced Combined Stress May Affect the Expressions of ABA Aldehyde Oxidase, Sucrose Synthase and Proline Metabolic Genes in Maize Seedlings

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Received: 24 October 2019; Accepted: 06 December 2019.

Abstract: The endoplasmic reticulum (ER) is an organelle in the cell where proteins are created and folded. Folding is a very elaborate process that is often interrupted by various biotic and abiotic stresses, leading to the formation of unfolded and misfolded proteins called ER stress. Dithiothreitol (DTT)-induced unfolded protein response (UPR) in endoplasmic reticulum (ER) has been recently reported in plants. Also, previous studies demonstrated that treatment with polyethylene glycol (PEG_{6000}) could stimulate water deficit in crops. However, further researches should be conducted to elucidate the molecular mechanism of ER stress response and the relationship between water deficiency and ER. In this study, we examined the expressions of *sucrose synthase* (*SuS*) gene, proline metabolic genes and *abscisic aldehyde oxidase (AAO3)* gene in maize seedlings that were subjected to DTT and PEG induced combined stresses by using quantitative real-time RT-PCR. Three weeks old detached maize seedlings were treated with or without DTT and PEG_{6000} for 12 h. The treatment with DTT increased about 2-fold the expression of gene encoding proline synthesis enzyme, *pyrroline-5-carboxylate synthetase* (*P5CS*) but no statistically affected the proline catabolism enzyme, *proline dehydrogenase* (*ProDH*) in comparison with un-treated seedlings. PEG treatment was also up-regulated *P5CS* while it was down-regulated *ProDH*. The relative expression levels of *SuS* and *AAO3* genes statistically enhanced about 2.5 fold under the DTT-induced ER stress. Likewise, the expression levels of *SuS* and *AAO3* genes were up-regulated in the detached seedlings exposed to PEG-induced water deficit. Conversely, the induced gene expressions were down-regulated under the combined stress, the DTT-induced ER stress and PEG-induced water deficit in comparison with the singular stress responses (DTT or PEG). The results indicated that the expressions of genes, related to the synthesis of some signal osmolyte compounds such as proline and sucrose can be suppressed when ER stress occurred under water deficiency in maize seedlings. The changes in the expressions of genes involved in osmolyte and ABA metabolism can be related to ER stress response as well as variations in water status.

Keywords: ER stress; water deficit; sucrose; proline; metabolism; ABA synthesis; gene expression

1 Introduction

As plants are sessile organisms, they can confront with a lot of environmental stresses such as drought, high temperature, salinity and chilling during their life cycle. These stresses can cause tissue dehydration, namely osmotic stress and the associated oxidative stress. Because of environmental stresses, the balance between protein folding demand and folding capacity in the endoplasmic reticulum (ER)

could be disturbed and thus unfolded or misfolded proteins could accumulate in the ER, leading ER stress [1]. Accumulation of unfolded or misfolded proteins activates a signalling pathway called the unfolded protein response (UPR), acting to relieve ER stress. Mechanism of UPR prevents abnormal maturation of proteins in the ER and, the mechanism leads to cell death if it becomes unsuccessful. In the UPR, genes encoding ER-resident molecular chaperones are co-operatively induced to cope with misfolded proteins in the ER [2]. Some studies showed that tunicamycin (TM) and dithiothreitol (DTT) elicited ER stress response in plants. Dithiothreitol generates ER stress by disrupting the redox conditions needed for the formation of disulfide bridges in proteins [1,3]. Dithiothreitol is a strong reducing agent that is also often used to promote reductive stress. It can cross membranes and prevent disulfide bond formation. A reductive stress induced by DTT treatment leads to an accumulation of misfolded proteins in the ER [4].

Many genes whose functions related to protection against reductive stress, not the ER and a part of the UPR, were up-regulated in response to treatment with DTT [5]. It was recorded that some genes are regulated in responding to ER stress [6]. Moreover, Arabidopsis bZIP28, an ER membrane-associated transcription factor (ER membrane-associated basic leucine zipper), was activated in response to ER stress induced by adverse environmental conditions or by exposure to ER stress agents such as TM and DTT [7]. Yu et al. [3] were conducted transcriptomic analysis to understand the mechanism of wheat response to ER stress in the seedlings exposed to DTT and they reported that 158 photosynthesis-related genes, 42 antioxidant enzyme genes, 318 plant hormone-related genes and 457 transcription factors (TFs) played vital roles in regulating wheat response to ER stress. Additionally, Harding et al. [8] demonstrated that amino acid biosynthesis genes were induced and amino acid metabolism including proline was upregulated in response to ER stress. Likewise, it was recorded that synthesis of some osmolyte compounds such as proline and soluble sugars (namely sucrose, glucose and fructose) increased in plants as responses to various environmental signals [9,10].

Osmolyte compounds allow dehydrated plant cells to withstand the dehydration by maintaining turgor, by acting an antioxidant role and by providing a redox homeostasis [9,10]. Proline accumulation due to increased synthesis and decreased degradation under abiotic and biotic stress conditions has been well documented in many plants [11]. Moreover, proline metabolism has an important role in ER stress tolerance and proline biosynthesis is critical for maintaining the intracellular redox environment and the UPR during ER stress [12]. On the other hand, the first two steps of proline biosynthesis are catalysed from glutamate by a single bifunctional enzyme, Δ^1-pyrroline-5-carboxylate synthase (P5CS), which produces glutamic-γ-semialdehyde (GSA). This GSA is spontaneously converted to pyrroline-5-carboxylate (P5C) that is reduced by P5C reductase (P5CR) to proline. Also, the proline accumulation depends on its degradation which is catalysed by proline dehydrogenase (ProDH), a mitochondrial enzyme [11]. Proline accumulation in plants exposed to environmental stresses is related to activation of the gene encoding P5CS, the key regulatory and rate-limiting enzyme in the biosynthesis [13]. The expressions of genes encoding *P5CS* increased while the *ProDH* expression decreased in maize exposed to water deficiency [14]. Likewise, in several plants, a strong correlation between proline accumulation by overexpression of the *P5CS* or by suppression of the *ProDH* gene and drought tolerance was demonstrated [15].

Soluble sugars act as signals regulating various processes associated with plant growth and development [16]. Sucrose synthase (SuS) playing a key role in sugar metabolism is a glycosyl transferase enzyme, primarily in sink tissues. SuS reversibly catalyses the cleavage of sucrose into fructose and, either uridine diphosphate glucose or adenosine diphosphate glucose. The products of sucrose cleavage by SuS are available for many metabolic pathways, such as energy production, primary-metabolite production, and the synthesis of complex carbohydrates [17]. In addition to meeting carbon requirement and energy for plant growth, soluble sugars can also regulate signals that control the transcription of genes related to the stress tolerance [10].

It has been also well known that abscisic acid (ABA) level increased in response to different stress factors in plants. ABA plays important role in many aspects of plant growth and development, including seed maturation and dormancy as well as adaptation to several environmental stresses. De nova synthesis

of ABA regulates these processes. Oxidation of abscisic aldehyde is the last step of ABA biosynthesis and is catalysed by abscisic aldehyde oxidase (E.C. 1.2.3.1) (AAO3) [18]. Drought stress induced ABA accumulation in the leaves and roots of *Arabidopsis thaliana* and *Pisum sativum* [19,20]. ABA biosynthesis-related genes were previously reported to be upregulated and ABA degradation-related genes were downregulated in response to heat stresses in Arabidopsis [21]. ER stress inducers such as DTT and TM induced ZmbZIP17 and translocation of ZmbZIP17 to the nucleus in *Zea mays* [22]. Furthermore, bZIP17 was probably involved in ABA-mediated ER stress response. However, whether bZIP17-mediated ABA response is directly linked to the ER stress response remains unclear [21]. Although several studies were conducted on the responses of plants to abiotic stresses, the role of ABA in ER stress response is still unknown.

As mentioned above, osmolyte compounds have a signal role in response to the abiotic stress factors and play an important role in protecting plants against hazardous effects of environmental stresses. Several studies thus far indicated that DTT application induced ER/oxidative stress in plants [6,23]. However, the effects of DTT-induced ER stress on the expressions of the genes encoding specific enzymes involved in osmolyte metabolism and ABA synthesis have not yet been documented in plants. In this context, this study was planned with the hypothesis that combined stresses, DTT-induced ER stress and PEG-induced water deficit stress can modulate the expressions of genes coding some enzymes involved in osmolyte and ABA metabolism in maize seedlings. Therefore, the present study was designed to evaluate the regulation of osmolyte metabolism and ABA biosynthesis in response to treatment with DTT in detached maize seedlings under water deficit stress.

2 Material and Methods

2.1 Plant Material and Growth Condition

Seeds of maize (*Zea mays* L.) cv. Akpınar obtained from the Black Sea Agricultural Research Institute, Turkey, were used as the plant material. The plants were grown in a growth chamber under controlled conditions (day/night temperature of 25/22°C, 60 ± 2% relative humidity and photosynthetic photon flux density of 400 µmol $m^{-2}s^{-1}$ with 16 h light and 8 h dark) in plastic pots (15 cm diameter, 20 cm depth) containing soil. When the seedlings had three fully expanded leaves, approximately after 21 days of growth, the seedlings were cut from 2 cm above the ground level and kept in distilled water for one hour to minimize the damage of water deficit as similar to Sezgin et al. [24]. DTT was used in the experiments as a chemical causing protein misfolded. The excised seedlings were also exposed to water deficit stress on PEG_{6000}. Therefore the excised seedlings were exposed to four different treatments for 12 h: (1) distilled water (Control treatment), (2) water deficit (-0.45 MPa) created by polyethylene glycol (PEG_{6000}) treatment (PEG treatment) (3) 20 mM DTT treatment (DTT treatment), and (4) DTT with by PEG_{6000} (DTT + PEG treatment). Water deficit was created by addition of PEG_{6000}, dissolved in the distilled water in increasing doses with an interval of 20 min until a final water potential of -0.45 MPa as similar to the method described by Sezgin et al. [24]. The DTT concentration was detected according to the indicators of water loss (leaf rolling and low leaf water potential) and the excised seedlings were immersed into the solution that is prepared at this concentration (20 mM DTT). The combined stress solution (DTT + PEG treatment) was prepared by dissolving 20 mM DTT in the PEG_{6000} solution. The leaves (third) were used for the gene expression analysis after the treatments for 12 h and kept at -80°C.

2.2 Gene Expression Levels

2.2.1 RNA Isolation and cDNA Synthesis

The leaves kept at -80°C were used for total RNA isolation. Frozen leaf segments (0.1 g) were crushed into a fine powder in a homogenizer with liquid nitrogen. Total RNA isolation was carried out by using a total RNA isolation kit (QIAGEN RNeasy Plant Mini Kit, Düsseldorf, Germany) (Cat. No: 74904) according to the manufacturer's protocols. The amount and purity of the RNA samples was measured at 260 nm by using a nanodrop spectrophotometer (Thermo Scientific, Nanodrop 2000, USA). cDNA

synthesis was conducted by using a high-capacity cDNA Reverse Transcription Kit 4368814, (Applied Biosystems, Waltham, USA) on the isolated total RNA samples (2000 ng RNA) according to the manufacturer's instructions. This cDNAs were used as templates for real-time PCR.

2.2.2 Quantitative Real Time (qRT)-PCR Analysis

PCR plates (BioRad, Hard- Shell PCR Plates Full C/C 10487009) having 96 wells were used. For each qPCR, 20 µl of total volume with gene specific primers were used, with 4 µl Supermix (5x HOT FIREPol Eva Green qPCR Supermix (08-36-00008, Solis Biodyne), 1 µl reverse and 1 µl forward primers, 1 µl cDNA sample and 13 µl nuclease-free water were added into each well. The analysis was performed on the CFX Connect Real Time PCR System (Bio-Rad). The qRT PCR protocol was modified according to Solis BioDyne's instructions: 95°C for 12 min, 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and a melt curve was held in 0.5°C increments from 60°C to 95°C. Each biological replication was analyzed as three technical replications, and the average technical errors were considered to be in the form of 0.5(± 1) Cq values. The findings were normalized according to the β-*actin* gene, and relative gene expression was presented. Primers of *P5CS, ProDH* acting role in proline metabolism, primer of *SuS* acting role sucrose biosynthesis and primer of *AAO3* acting role in ABA biosynthesis pathway were listed in Tab. 1.

Table 1: The sequences of specific primers used for the qRT-PCR analysis

Target gene	NCBI accession no	Primer sequences
Actin1	NM_001155179.1	ACT1Zm-F: "GAA GAT CAC CCT GTG CTG CT"
		ACT1Zm-R: "ACC AGT TGT TCG CCC ACT AG"
P5CS	DQ864376.1	P5CSZm-F: "AACATCTTGCCCTCTGGGTG"
		P5CSZm-R: "CCATTGCCACTTCGAACTGC"
ProDH	NM_001154105.1	ProDHZm-F: "TCAGCAAGTACCTGCCGTAC"
		ProDHZm-R: "ACCCTCCTCACCAACTCCTT"
SuS	NM_001111941.1	SuSZm-F: "GAATGCTTCAGCGCCATCAG"
		SuSZm-R: "CTTCCTGAGCAGCACGAAGA"
AAO3	NM_001111838.1	AAO3Zm-F: "CCTGACAAGAAGCGTGTCCT"
		AAO3Zm-R: "GAATGTGACGGCGGATTTCG"

2.3 Statistical Analysis

All experiments were performed six times with six biological replicates. All results were presented as means ± standard deviation. The statistical analysis was conducted using Duncan multiple comparison test (One-way ANOVA) [25] using the SPSS for Microsoft Windows (Ver. 23.0, SPSS Inc., USA) to evaluate if the means were significantly different or not. The significance level among all treatments was accepted to be 5% ($p < 0.05$).

For qRT PCR analysis, the relative gene expression level was analyzed by Bio-Rad CFX Manager 3.1. Expression levels were assayed by the SPSS software.

3 Results

3.1 The Relative Expression Levels of Genes Involved in Osmolyte Metabolism

The relative expression of *P5CS* involved in proline biosynthesis was presented in Fig. 1. The expression of *P5CS* gene was up-regulated by water stress (PEG). The expression was more induced in the DTT treatment. Conversely, under the combined stress condition (DTT + PEG treatment), P5CS expression was down-regulated in comparison with the other treatments including the Control treatment.

As for the expression of the *ProDH* gene that is responsible for proline degradation, the expression

level reduced in the PEG-treated seedlings as compared to the Control treatment. *ProDH* gene expression no statistically changed in the seedlings treated with DTT. Similar to the *P5CS* expression, the level of *ProDH* gene expression decreased in the DTT + PEG-treated seedlings in comparison the other treatments (Fig. 2).

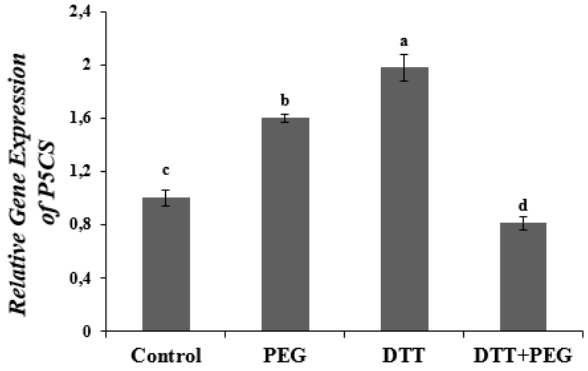

Figure 1: The changes in the relative expression levels of *P5CS* gene in detached maize seedlings. The data show means ± SD of six replicates. Different letters indicate significant differences ($p < 0.05$)

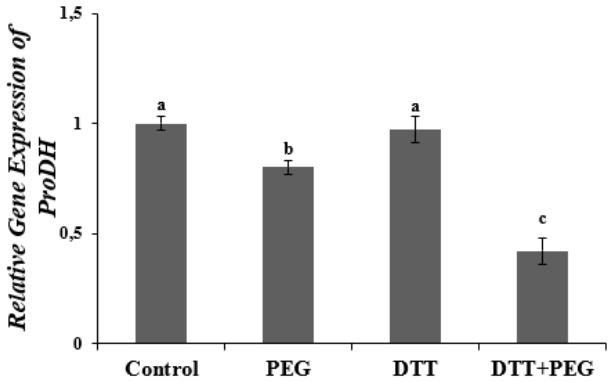

Figure 2: The changes in the relative expression levels of *ProDH* gene in detached maize seedlings. The data show means ± SD of six replicates. Different letters indicate significant differences ($p < 0.05$)

It was found that the relative gene expression of *SuS* increased under the singular stress conditions. However, the treatment with DTT + PEG decreased the expression level of *SuS* gene in comparison with the singular stresses not the control treatment (Fig. 3).

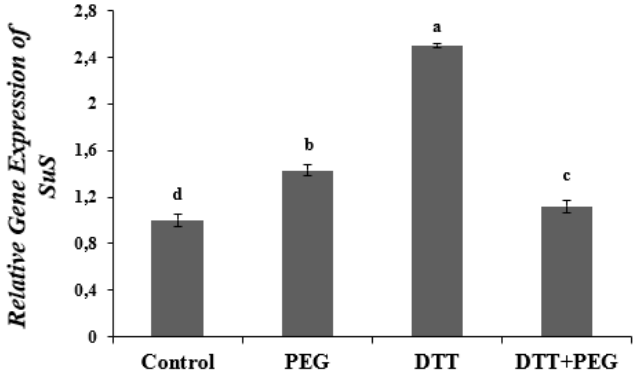

Figure 3: The changes in the relative expression levels of *SuS* gene in detached maize seedlings. The data show means ± SD of six replicates. Different letters indicate significant differences ($p < 0.05$)

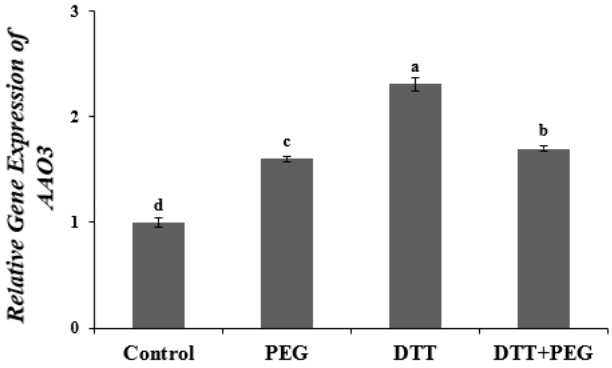

Figure 4: The changes in the relative expression levels of *AAO3* gene in detached maize seedlings. The data show means ± SD of six replicates. Different letters indicate significant differences ($p < 0.05$)

3.2 The Relative Expression Levels of the Gene Involved in ABA Biosynthesis

As shown in Fig. 4, the expression level of *abscisic aldehyde oxidase (AAO3)* gene in maize seedlings increased under water stress. Also, *AAO3* relative gene expression was more upregulated in the seedlings treated with DTT in comparison with the PEG treatment. There was a decrease in the expression level of the *AAO3* gene in the seedlings that are exposed to combined stress in comparison with the DTT treatment (Fig. 4).

4 Discussion

The UPR was one of the protein quality control steps for ensuring the correct protein folding in the ER [26]. The association of UPR with many abiotic stress and biotic stress responses in plants has been reported in previous studies [27]. Moreover, it has been known that in the natural ecosystem, plants have been simultaneously exposed to multiple stresses [21]. The ER is highly sensitive to the effects of stress on intracellular energy levels, oxidative status, and calcium ion concentrations [3]. However, it is not yet clear how ER stress affect the expressions of genes related do osmolyte metabolism and ABA synthesis in plants. In this study, we examined the relative expressions of three genes involved in osmolyte metabolism and a gene related to a key enzyme of ABA biosynthesis in the detached maize seedlings under DTT and PEG-induced combined stress conditions using real-time RT-PCR.

The UPR is usually induced in the laboratory by treatment with ER stress agents, such as DTT or TM [1]. So, in the present study, we used DTT (20 mM) to induce ER stress response in the detached maize seedlings. Here, we should mention that as indicators of water status, leaf rolling is induced and, water loss was observed in the seedlings exposed to 20 mM DTT. Although there have been several studies of proline protection against various types of environmental stress, very little is known about the role of proline in ER stress response. The results showed that the ER stress, induced by treatment with DTT increased the relative expressions of genes encoding *P5CS*, proline synthesis enzyme, but did not statistically affect *ProDH*, proline catabolism enzyme, in comparison with un-treated seedlings. PEG treatment also up-regulated *P5CS* while it down-regulated *ProDH*. Indeed, it was reported that the expressions of genes encoding *P5CS* increased while *ProDH* expression decreased in maize seedlings exposed to water deficiency [14]. Also, Seki et al. [15] reported that a strong correlation between proline accumulation and drought tolerance was induced by overexpression of *P5CS* or by suppression of the *ProDH* gene. In a current study, it was reported that proline limitation negatively impacted antioxidant defence systems and the UPR during ER stress recovery [12]. Also, we found that the expression levels of *P5CS* and *ProDH* genes were down-regulated under the combined stress in comparison with the Control treatment and the seedlings exposed to singular stresses (DTT or PEG treatments). Therefore, this result indicates that proline metabolism may be slowed down under ER stress response combined with water deficiency.

Sugar metabolism play important roles in the osmotic adjustment process, cryoprotection and signalling in plants. In addition, soluble sugars, especially when they are present at higher concentrations, might act as ROS scavengers by themselves. Soluble sugars might either directly detoxify ROS in chloroplasts and vacuoles or indirectly stimulate the classic antioxidative defence systems [28]. It was known that SuS and sucrose-phosphate synthase regulated sucrose metabolism in plants [29]. SuS plays a central role in secondary metabolism of sucrose. Our results showed that the relative expression levels of *SuS* gene statistically enhanced under the PEG-induced water deficit and more increased under the DTT-induced ER stress. According to our best of knowledge, our current study on the changes in osmolyte metabolism during ER stress may be first record in the literature. On the other hand, our finding on *SuS* gene expression is consistent with the findings of a previous report that heat shock protein (PpHsp16.4) was strongly induced by treatment with DTT (10 mM) to provide abiotic stress tolerance in *Physcomitrella patens* [23]. Likewise, a previous study showed that ER stress response genes were significantly up-regulated in leaves of *Jatropha curcas* L. under drought stress [30]. While the expression level of *SuS* was induced under the singular stress conditions (DTT and PEG treatments), the relative expression of *SuS* was down-regulated under the combined stress condition (DTT + PEG treatment). These findings suggested that the expressions of genes related to the synthesis of some signal osmolyte compounds such as proline and sucrose can be suppressed in the detached maize seedlings exposed to combined stresses, water deficit plus ER stress.

It has been well known that ABA regulates many aspects of plant growth and development and plays a central role in the response to environmental stresses. The genes related to ABA biosynthesis were upregulated and ABA degradation-related genes were downregulated by high temperature in Arabidopsis [31]. Similarly, heat treatment increased the level of ABA in rice [32]. Despite the increasing number of reports on ABA and heat stress, only a few studies have been conducted to investigate the effect of ABA on ER stress [21]. ABA level also increased under environmental stress conditions in plants. ABA in roots subjected to water loss is thought to be a signal that is transported to the leaves, and to be caused the stomatal closure [18]. On the other hand, major genes encoding the enzymes that catalyse the different steps of ABA biosynthesis have been identified and the biosynthetic pathway elucidated in plants [33-35]. It was reported that the abscisic aldehyde oxidase gene products catalyse the final step in abscisic acid (ABA) biosynthesis in plants [36]. As similar to *SuS* gene expression, we found that the expression level of *AAO3* statistically enhanced in the detached seedlings exposed to the singular stresses (ER stress or water deficit). Moreover, the increase in the *AAO3* expression was higher in the DTT treatment than that of PEG treatment. Up-regulated *AAO3* gene expression pointed out that ABA content could be enhanced in the detached seedlings that are subjected to DTT-induced ER stress or PEG-induced water deficit. Namely, *AAO3* gene expression was high in the seedlings exposed to PEG-mediated water stress and DTT-induced ER stress and the high gene expressions might be related to be alleviation of the stress damages. Our findings are similar to those reported by Seo et al. [18] who reported that *AAO3* gene expression was rapidly induced in dehydrated rosette leaves of *Arabidopsis thaliana*. On the contrary, the induced gene expression levels were down-regulated in the DTT + PEG-treated seedlings. As based of this finding we can say that *AAO3*, ABA synthesis gene, could be down-regulated under DTT and PEG-induced combined stress conditions.

Consequently, proline metabolic genes (*P5CS* and *ProDH*), *SuS* and *AAO3* genes were down-regulated in the maize seedlings exposed to the combined stress that is provided by DTT-induced ER stress and PEG-induced water deficit stress. Therefore, we concluded that the expressions of genes, related to the synthesis of some signal osmolyte compounds such as proline and sucrose can be suppressed when ER stress occurred under water deficiency in maize seedlings. Moreover, the changes in the expressions of genes involved in osmolyte and ABA metabolism can be related to ER stress response and as well as variations in water status. The DTT-induced variations of the gene expression might be dose-specific [37]. We can propose that the modified ER stress response through DTT application can be partly responsible for the changes in gene expression related to some signal compounds. This study provides information on the coordination of ER stress responses with stress tolerance related to osmolyte

and ABA metabolism in maize. On the other hand, Gasch et al. [5] recorded that many genes were up-regulated in response to DTT treatment whose functions seem unrelated to the ER and do not form part of the UPR. They also reported that these gene products were likely to function in protection against reductive stress [4]. As based of this record, we can also say that the changes in the gene expressions, in the present study, involved in osmolyte metabolism and ABA synthesis, may be connected with reductive stress or high reactive oxygen species. So, further research is needed to elucidate the modifications in gene expressions during the ER stress responses.

Conflict of Interest: The authors declare that they have no conflict of interest to report regarding the present study.

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