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Research Article

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Altered Expression of Superoxid Dismutase (SOD) Isoforms Is Necesary For Better Performance of A Recently Developed Drought-Tolerant Mutant of Rice Under Dehydration Stress

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Abstract
Drought is one of the most important abiotic stresses, which annually reduces agriculture production worldwide. With regard to the occurrence of successive drought stress in the life period of crop plants, dealing with the stress, particularly in rice growing regions needs special attention. Therefore, this study aimed to investigate and to compare a recently-developed drought-tolerant mutant line under dehydration stress with its original parental cultivar (Neda). Mutant line performed better than its original parental cultivar Neda at seedling and whole plant stages under dehydration and drought stresses, respectively. For gene expression analysis, dehydration stress was mimicked by polyethylene glycol 6000 (osmotic pressure of -6 bar), and sampling was done at 0, 12, 24 and 36 hours after the stress, and expression of two isoforms of superoxide dismutase (SOD) gene was evaluated by real-time quantitative PCR method. Assessment of relative expression of superoxide dismutase isoform OsMnSOD at different times after stress showed that the expression of this gene in the parental cultivar was not significantly altered after the stress, while in the mutant line MT149 it was significantly decreased within early hours after stress but was significantly increased up to 11-fold within 24 hours after stress, and the expression of another isoform of superoxide dismutase, OsCu-ZnSOD, in mutant line MT149 also was first decreased and then increased up to 9-fold within 24 hours after stress. On the basis of the results of this research it can be concluded that probably one of the reasons for higher drought/dehydration tolerance of the mutant MT149 compared to Neda, is the increased expression of both isoforms of OsMnSOD and OsCu-ZnSOD within first 24 hours of exposing to the stress, making it possible to overcome the toxic effects of reactive oxygen species (ROS).

Keywords: Dehydration, Drought, Gene expression, Rice, SOD

Introduction

Rice (*Oryza sativa L.*) is a cereal crop of high socioeconomic value and a staple food for over half of the world's population. Iran is one of the major rice importers in the world. According to the UN Food and Agriculture Organization (FAO), the total volume of rice production in Iran in 2013 was 2.9 million tons and about 1.9 million tons was imported at a cost of over 2.3 billion dollars [1]. The area under rice cultivation in Iran is about 570,000 hectares and more than 80 percent of land under the rice cultivation is in the Guilan, Mazandaran and Golestan Provinces [1]. Therefore with regard to the water shortage and limited arable area in Iran the rice production per unit area must be increased. Since the large part of the land under cultivation in the world and Iran locates in the semi-arid climatic conditions, considering new methods is needed to reduce the yield losses due to drought stress.

Drought is one of the great abiotic stresses that strictly affects and reduces the yield and productivity of food crops worldwide. Response to drought stress in plants is a complex trait involving changes in plant morphology, physiology and metabolism [2]. When plants face periods without rainfall during life cycle, they use a convened redirection of metabolism related to different and sometimes supplementary physiological and biochemical mechanisms, such as stomatal closure, leaf rolling, the abscisic acid sig-

naling pathway, decrease in the photosynthetic and transpiration rates, solute reposition, the production of free radicals and characteristic metabolites, and changes in antioxidant enzymes and their gene expression levels [3]. In another hand, drought stress leads to accumulation of reactive oxygen species (ROS). Reactive oxygen species (ROS) molecules such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H2O2) and hydroxyl radical (OH-) are highly reactive, toxic intermediates of oxygen metabolism [4]. In plants, ROS are common byproducts of several metabolic pathways and are also produced under stress conditions in diverse cellular compartments, including chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum (ER), and plasma membranes [5, 6]. Majority of the oxidative stresses damage the plant cells via the formation of ROS which are produced directly by stimulation of O2 or by transmission of one, two, or three electrons to O2. The activation of oxygen can occur via two different mechanisms. The first is the absorption of adequate energy to contrary the spin on one of the unpaired electrons to form the singlet state, in which the two electrons have opposite spins. The second path leading to production of activated oxygen is the stepwise reduction of oxygen to form ROS: superoxide => hydrogen peroxide => hydroxyl radicals [7].

High levels of ROS can damage macromolecular structures in cells, lipids, proteins and DNA, and if are scavenged at the right time it can cause cell death [8, 9, 10]. To support the structure and operation of plant cells from the noxious effects of ROS, a complex antioxidant system is activated. This system include three ingredients: (1) lipid soluble and membrane-associated tocopherols; (2) water soluble reducing compounds such as ascorbate (AsA) and glutathione (GSH), and (3) antioxidant enzymes [4]. There are many non-enzymatic ROS scavenging antioxidants that include ascorbic acid (vitamin C), gluthation, proline, α-tocopherols (vitamin E), carotenoids (Car), flavonoids and alkaloids [11]. Primary reactive oxygen intermediates (ROI)-scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) [12]. The SOD antioxidant enzyme which catalyzes the dismutation of the superoxide anion (O2•-) into hydrogen peroxide and molecular oxygen, is one of the most important primary defense mechanisms against oxidative stress and plays an acute role in survival of plants under environmental stresses [3]. This enzyme is present in all aerobic organisms and cell compartments procumbent to oxidative stress. The SOD enzymes, based on their cofactors are classified into three isoforms which are localized in different cellular organelles: Fe-SOD (localized to chloroplasts), Mn-SOD (localized to mitochondria), and Cu/Zn-SOD (localized to chloroplasts, peroxisomes, and cytosol) [13].

A notable increase in the activity and/or expression of SOD in plants under drought stress has been observed in various plant species, such as common bean, cowpea, transgenic rice plants, and sweet potato [14-17]. The rice genome encodes 7 SOD genes. In general, SODs in plants are encoded by small gene families [13]. These isoforms are explicit differentially in response to various stresses [18] which suggests that each isoform has a divergent practice in plant cells and that convened regulation of multiplex isoforms is required for fine control of the cellular ROS levels [6]. Among the multiple SOD isoforms localized in different subcellular compartments, the transcript levels of *OsMnSOD* and *OsCu-ZnSOD* were evaluated. Recently, a rice mutant line (named MT149) was isolated and introduced via screening of a mutant population resulted from mutagenesis of cv. Neda with the ethyl methane sulfonate (EMS), which in initial evaluations revealed drought-stress tolerance in both seedling and adult plant stages [19]. Therefore, the purpose of this study was to evaluate the effect of dhydration stress on the expression of two isoforms of superoxide dismutase (viz. *OsMnSOD* and *OsCu-ZnSOD*) in the mutant line MT149 compared to its parental line (Neda).

Materials and Methods

Plant Materials, Growth Conditions And Stress Treatment

The plant material used in this study consists of cultivar Neda and MT149 mutant line. cultivar Neda is a high-yielding, semi-dwarf and non-tolerant to drought, and MT149 mutant line was isolated and introduced recently via screening of a mutant population resulted from mutagenesis of rice (cultivar Neda) with the chemical mutagen ethyl methane sulfonate (EMS), which in initial evaluations showed drought-stress tolerance in both seedling and adult plant stages [19].

Seeds of two genotypes were collected and surface-sterilized and allowed to germinate in sterilized Petri dishes on sterilized filter paper soaked with dH_2O at 28 ^{0}C for 5 days. Germinated seeds were transferred to plastic glasses containing Yoshida solution and grown in a growth chamber under controlled conditions, using 28°C day/25°C night temperatures, 16-h light/8-h dark cycles for 2 weeks[20]. The experiment was performed according to a completely randomized design (CRD) with three replications, in which the two genotypes were studied at two dehydration stress levels (normal and -6 bar). Dhydration stress treatment was performed with polyethylene glycol 6000 (osmotic pressure of -6 bar). The calculation of osmotic pressure was done on the basis of Michel and Kaufmann formula [21]. Tissue sampling was done at 0, 12, 24 and 36 hours after the stress. Leaf tissues were then harvested, frozen in liquid nitrogen, and stored at -70°C for further analysis.

Drought response of the two genotypes also was evaluated at whole plant stage in paddy field. 30-day-old seedlings of the two genotypes were transplantd in two separate blocks (one block for well-water treatment amd one block for low-water treatmet) with three replicates. The low-water treatment was started from early tillering stage (2 weeks after transplanting) to harvest time. Following traits were measured at proper times: plant height (PH, cm), days to heading (HD, days), panicle length (PL, cm), tiller number (TN), kernel number per panicle (KN), sterile kernels per panicle (SK), plant yield (PY, g/plant), biomass weight (BW, g/plant).

RNA Extraction, cDNA Synthesis and qRT-PCR

For each genotype, a bulk of approximately 50 mg of plant leaf in the three replications was homogenized in liquid nitrogen and total RNA was extracted using the RNA Reagent kit (RNX-plus) according to manufacturer's instructions. Quantity and quality of the isolated RNA was evaluated by spectrophotometry and electrophoresis in 1% agarose gel, respectively. Before synthesizing cDNA, DNase I treatment was conducted at 37 ºC for 30 min followed by incubation at 65 ºC for 15 min. Five micrograms of each RNA sample was incubated with 1 U *DNase I* to remove residual DNA contamination. Then, cDNA was synthesized from *DNase I*- treated RNA using a commercial cDNA synthesis Kit (Aria Tous Co., Iran).

For the assessment of differential expression of two isoforms of superoxide dismutase gene (with accession numbers of L19436.1 and DQ058108.1 for *OsMnSOD* and *OsCu-ZnSOD*, respectively), two specific primers were designed for each gene. Primer sequences for *OsMnSOD* were 5´-ACTGCTTGATGTTGTCTGAAGG-3´ for the forward primer and 5´-GCATTAGTCCACCTCTACGC-3´ for the reverse primer. Primer sequences for OsCu-ZnSOD were 5´- GGGATCATCGGACTTCAAGG-3´ for the forward primer and 5´- GAGATCAGGTGCTTATTGTAGAATC-3´ for the reverse primer. A house-keeping gene, *β-Actin*, was used as internal control in quantitative real-time PCR (qRT-PCR). The *β-Actin* primer sequences were 5´-GTCGGTGAAGGGGACTTACA-3´ for the forward primer and 5´-TTCATACAGCAGGCAAGCAC-3´ for the reverse primer. qRT-PCR was performed on an ABI real-time PCR system (Applied Biosystems) using SYBR Green I Kit (Aria Tous Co., Iran) under the following conditions: the cycling program consisted of a denaturing step of 94 °C for 15 min, followed by 45 cycles of 94 °C for 15 sec, 61 °C (for both *OsMnSOD* and *OsCu-ZnSOD* isoforms) or 56 °C (for *OsActin* gene) for 35 sec and an elongation step of 45 sec at 72 oC.

Statistical Analysis

Analysis of variance (ANOVA) was done using SPSS v.16. Mean comparisons was done by LSD test or Ducncan's multiple range test. In real-time PCR analysis, the calculation of threshold cycle (CT) in each cDNA sample was conducted using the software LINREG. The significant test of gene expression data was done by the Pfaffl altered method using the software REST. The changes in relative expression of two genes in response to dehydration stress were quantified using the 2-ΔΔCt method, where $\Delta C_t = C_T$ (Target) $-C_T$ (β-Actin) [22, 23].

Results and Discussion

Drought Stress Effects At Seedling Stage

Evaluation of the effect of dehydration treatment showed that under dehydration stress condition mimicked by PEG, the shoot expansion rate was significantly lower than that under non-stress condition (Figure 1 left). It was reported that drought stress affected directly or indirectly morphological and physiological statuses of plants [24, 25]. Also reported that growth parameters of different rice cultivars at vegetative stage were reduced under PEG-mimicked drought stress.

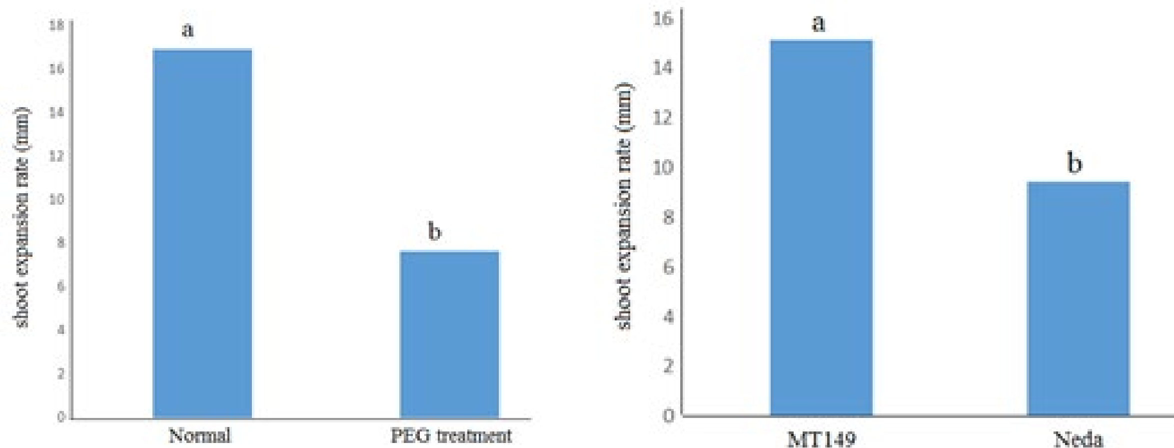

Figure 1: PEG-induced dehydration effect on shoot expansion rate: (Left) Comparison of treatment effects; (Right) comparison of two rice lines. MT149: tolerant mutant line; Neda: non-tolerant line. Note: histograms with different letters have significant mean differences.

Comparison of two lines under dehydration stress showed that mutant line MT149 (drought tolerant) had higher shoot expansion rate compared to parental non-tolerant line Neda (Figure 1 right). Some of parameters in rice are considerably affected by water deficit. Length growth of plant is one of important parameters which is significantly affected by drought stress. Several studies showed that plant growth was decreased after water deficit. For example, it was reported that drought stress directly affected rice plant growth and reduced plant height and number of tillers per plant [26]. The researchers denoted that this was occurred because plant could not absorb water from soil under drought stress resulting in unavailability of essential elements for plant that decreases cell division rate and enlargement. Yang and reported that drought stress reduced grain yield, growth of stem, leaf and root, and finally reduced plant height [27, 28]. A study reported that the most importan t consequences of drought in crop plants are the reduced cell

division rate, reduced stem length and reduced root growth [29]. Reported significant reduction of rice plant height under drought stress condition in the field. More recently it was reported a strong negative correlation between PEG concentration and shoot length [25].

Drought Response At Whole Plant Stage

Drought response of the two genotypes also was studied at whole plant stage in the paddy field under well-water and low-water conditions. As seen in Table 1, two genotypes mainly showed differential responses to drought stress in the paddy field. Plant height (PH) and panicle length (PL) in mutant MT149 did not change under drought while that of Neda was significantly reduced (-4.7% and -3.7%, respectively). Number of sterile kernels (SK) as an obvious criterion of drought damage at reproductive stage, in the case of mutant line MT149 wasn't significantly inreased under drought stress (2.9%) while that of Neda significantly increased (6.3%). Tiller number (TN) and plant yield (PY) in both genotypes were reduced under drought stress but they were more reduced in Neda relative to MT149 (-15.2% vs. -6.6% and -16.7% vs. -11.2%, respectively). The biomass weight (BW) response adhered from PY in two genotypes.

Table 1: Genotype Responses To Drought Stress At The Paddy Field

Genotype	Condition	PY	PH	HD	PL	TN	KN	SK	BW
Neda	Well-watered	43.2[a]	97.0[a]	92.9[a]	24.1[a]	17.1[a]	103.4[a]	10.5[a]	188.6[a]
	Low-watered	36[b]	92.4[b]	85.5[b]	23.2[a]	14.5[b]	102.6[a]	16.9[b]	164.6[b]
MT149	Well-watered	45.7[a]	94.7[a]	92.5[a]	24.5[a]	18.3[a]	104.1[a]	10.3[a]	223.6[a]
	Low-watered	40.6[b]	93.4[a]	86.7[b]	24.1[a]	17.1[a]	105.3[a]	13.5[a]	197.8[b]
Change under stress (%)	Neda	-16.7**	-4.7*	-8.0*	-3.7*	-15.2**	-0.8[ns]	6.3*	-12.7*
	MT149	-11.2*	-1.4[ns]	-6.3*	-1.6[ns]	-6.6*	1.2[ns]	2.9[ns]	-11.5*

Means with common letters have not significant differences. ns, * and **: indicate non-significant difference, significant difference at 5% level and significant difference at 1% level. PY: plant yield (g/plant), PH: plant height (cm), HD: days to heading, PL: panicle length (cm), TN: tiller number, KN: kernel number, SK: sterile kernels, BW: biomass weight (g).

Gene Expression Under Dehydration Stress

Among various abiotic stresses, drought stress is the most important limiting factor for crop plants. Exposing to water deficit in different growth stages causes different developmental and physiological changes, which all these changes are resulted from fluctuations in expression level of multiple genes. The first defensive mechanism of plants against reactive oxygen species (ROS) is superoxide dismutase enzyme [30]. In this experiment, the gene expression of two isoforms of superoxide dismutase (*MnSOD* and *Cu-ZnSOD*) was studied. As shown in Table 2, the *MnSOD* expression in Neda (non-tolerant line) after dehydration stress showed a subtle decrease within early hours after stress (12 h after dehydration), and then showed a little increase in gene expression within 24 hours after stress, but mutant line (tolerant) showed at first a strong decrease and then (24 hours after the stress) a strong increase in gene expression (up to 11-fold). This result clearly shows that *MnSOD* expression was reduced in tolerant line earlier than non-tolerant one. This alteration may be act as an awareness alarm for other sensory and signaling components of the cell that are sensitive to early excess of free oxygen radicals. After this early shock, 24 hours after dehydration stress the *MnSOD* is significantly activated to detoxify free oxygen radicals in muatant line MT149.

Table 2: *MnSOD* Isoform Expression In Two Genotypes Under Dehydration Stress With PEG at Different Times

Line	Time (hrs)	Relative expression	95% confidence	$P(H_1)$	Result of expression
Neda	12	0.526	0.13-2.79	0.361	No significant change
	24	0.338	0.23-0.56	0.056	No significant change
	36	1.64	0.69-4.70	0.303	No significant change
MT149	12	0.069	0.04-0.10	0.000	significant reduction in expression
	24	10.81	6.15-29.27	0.000	significant increase in expression
	36	0.329	0.20-0.50	0.056	No significant change

Moreover, in Figure 2 the changes in gene expression pattern in two genotypes is shown. As seen, Neda line after the stress did not show significant changes in gene expression, but in mutant line at first, gene expression was decreased and then was increased about 11 times, which indicates that mutant line likely have more tolerance against dehydration due to increasing in the MnSOD gene expression.

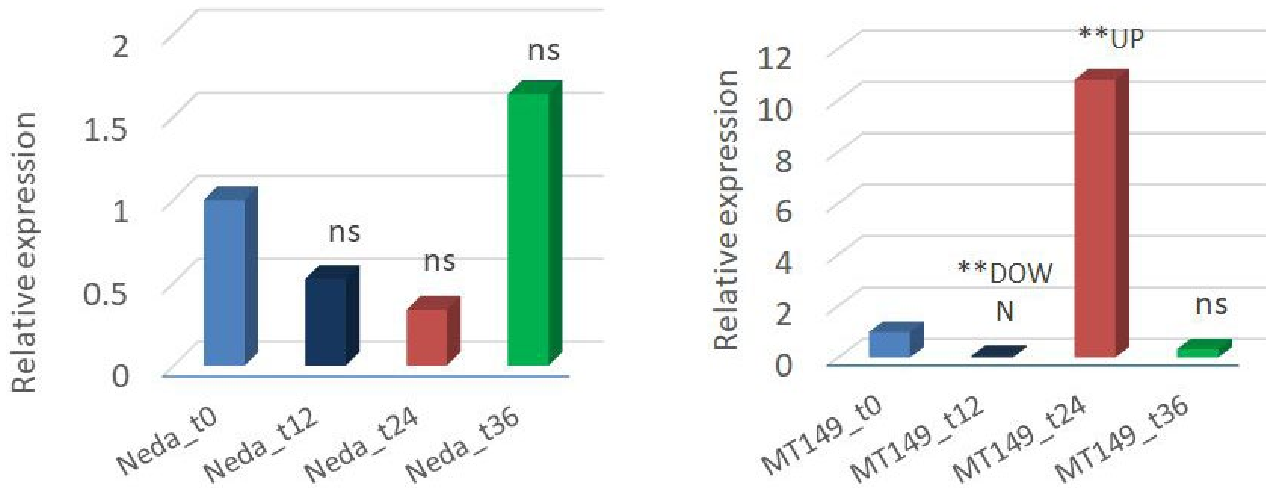

Figure 2: The changes in expression level of *MnSOD* isoform at different times of dehydration stress : (right) MT149 mutant, (left) parental line Neda.

The expression of Cu-ZnSOD isoform at dehydration stress condition was also investigated and as shown in Table 3, in Neda line the gene expression was not changed after the stress, while in mutant line it was changed, so that at first (after 12 hours) gene expression was decreased and then after 24 hours it was increased.

Table 3: *Cu-ZnSOD* isoform expression in two genotypes at different times after dehydration stress with PEG

Line	Time (hrs)	Relative expression	95% confidence	$P(H_1)$	Result of expression
Neda	12	0.198	0.03-1.61	0.168	No significant change
	24	0.863	0.54-1.32	0.325	No significant change
	36	1.65	0.73-3.96	0.220	No significant change
MT149	12	0.056	0.03-0.09	0.037	significant reduction in expression
	24	9.404	2.05-34.80	0.000	significant increase in expression
	36	0.426	0.25-0.97	0.166	No significant change

Also, in Figure 3 is shown the changes in gene expression pattern in two genotypes. As seen, relative gene expression in Neda after the stress had no significant changes, but in mutant line, at first, gene expression was decreased and then increased more than 9 times.

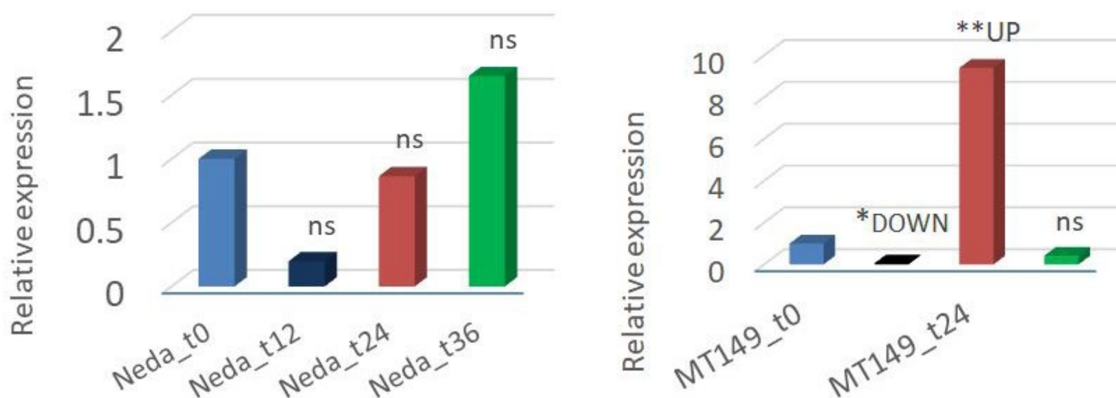

Figure 3: The changes in expression level of Cu-ZnSOD isoform at different times of dehydration stress: (right) MT149 mutant, (left) parental line Neda.

In general, the amount of antioxidants is correlated with the amount of tolerance to stresses and whatever the activity of antioxidants is more, the damage resulting from stresses to cells will be less [6, 31, 32]. Studies show that tolerant varieties to environmental stresses can stand environmental stresses through the induction of antioxidant defense systems. Therefore, there is a relation between antioxidant defense systems and stress conditions [33, 34]. It also was observed that transgenic plants that produce more antioxidant enzymes, have better tolerance to environmental stresses. Accumulation of metabolites and changes in enzymes activity such as catalase and peroxidase, may be related to plant`s potential to cope with the harmful effects of environmental stresses in the different physiological and morphological processes [35, 36].

As the results of this research showed, the expression of two *MnSOD* and *Cu-ZnSOD* isoforms in mutant line (MT149) within 24 hours after the onset of dehydration, significantly was increased, which is consistent with the results of other studies [37]. For example, also reported that the expression of *Cu-ZnSOD* and *MnSOD* genes showed a significant increase in plant tissues exposed to drought. One study showed that the activity of antioxidant enzymes in rice drought-susceptible varieties under drought is severely reduced, but in drought-resistant varieties their activity increased [34, 38]. Also reported the enhacement of *Cu-ZnSOD* expression in leavs of triticale under drought stress.These results along with our findings suggest that increasing in activity of antioxidant enzyme systems is essential to achieve drought tolerance. This is despite the fact that with giving drought stress by PEG in growth stage in the Hindi varieties of rice, reported that SOD activity in the leaf tissues of susceptible genotypes decreased, but in tolerant genotypes, the activity of the enzyme was unchanged [39].

After transferring the *MnSOD* gene to drought sensitive rice varieties, reported that the expression of this gene in sensitive plants increased and caused increasing the tolerance of the plants to drought. In addition, they reported that SOD can protect PSII from superoxide induced by stress [40]. Others also emphasized on the defense role of SOD isoforms in abiotic stress tolerance including drought stress [34, 41]. Recently, via a comprehensive analysis suggested the SOD as a selection criterion for triticale grain yield under drought stress. Interestingly, the application of plant-derived SOD was suggested as a health-enhancing factor for mammals [42].

Our results suggest that the reason for higher drought tolerance of mutant line MT149 compared to parental original line is likely higher expression of SOD isoforms under drought/dehydration stress. In contrast, reduction in the gene expression in Neda (non-tolerant line) probably causes its susceptibility to drought stress which is probably due to inhibition of production of enzymes or changes in the accumulation of enzymatic subunits under stress condition. In another side, increased expression of the two genes in mutant line probably is due to desire mutations in regulatory system of the genes so that binding of transcription factors to promoter regions enhances their transcription rate [43]. after studying on promoter regions of 25,000 human genes reported that probability of mutation occurrence in regulatory regions of protein encoding genes is significantly high. Also, other studies showed that mutation in intragenic or regulatory regions may affect binding of transcription factors to DNA or even on mRNA splicing [44, 45].

Conclusions

Based on the obtained results it can be concluded that better performance of the mutant line MT149 than its parental line (Neda) under drought/dehydration conditions and its higher tolerance is more likely due to the increased expression of both isoforms of *OsMnSOD* and *OsCu-ZnSOD* within first 24 hours of exposing to the stress, making it possible to overcome the toxic effects of reactive oxygen species (ROS) which usually is generated under stress.

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Conflicts of Interest

The authors declare no conflict of interest.

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