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Expression analysis of five critical transcription factors (TFs) *OsbHLH148*, *OsbZIP72*, *OsMYB2*, *OsNAC6* and *TRAB1* in response to drought stress in contrasting Iranian rice genotypes.

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Abstract

Drought stress causes great damage to the rice cultivation all over the world. Specific transcription factors (TFs) can regulate the expression of stress-related genes. In this research, we investigated the effect of drought stress on the expression of five specific transcription factors *OsbHLH148*, *OsbZIP72*, *OsMYB2*, *OsNAC6* and *TRAB1* at vegetative and reproductive stages in two Iranian rice cultivars with drought-sensitive and tolerant backgrounds; Hashemi and Neda. Using a real-time quantitative PCR (qPCR) approach, this study revealed that the expression of *OsbZIP72*, *OsMYB2* and *OsNAC6* were increased significantly in the Hashemi cultivar under drought stress at the vegetative stage. It seems that these three genes paly their roles in the drought sensitive cultivar Hashemi at the vegetative stage and do not play any role at the reproductive stage as the most sensitive stage to drought stress. The expression of *TRAB1* was increased in Hashemi cultivar at the reproductive stage, while the expression of *TRAB1* was decreased in Neda cultivar at the vegetative stage. This indicates that the expression of *TRAB1* could respond sensitively to drought stress at the vegetative stage. Furthermore, there were statistically significant increases in expression of *OsbHLH148*, *OsbZIP72*, *OsMYB2*, *OsNAC6* and *TRAB1* in Neda (tolerant) cultivar at the reproductive stage. Therefore, our study suggests that these five genes might be involved in drought tolerance of this cultivar to drought stress at the reproductive stage. Thus, they could be used as viable candidate TFs to develop additional varieties of drought-tolerant transgenic rice.

Keywords: Abiotic stress, desiccation tolerance, Transcript analysis, Growth stage, *Oryza sativa*, qPCR.
Abbreviations: bHLH_basic helix-loop-helix, bZIP_basic leucine zipper, *MYB*_myeloblastosis, NAC_ nam, ataf, and cuc, TRAB_transcription factor responsible for ABA regulation.

Introduction

Plants are exposed to various types of environmental stressors during their life cycle. Abiotic and biotic stresses cause major damages in crop productivity all over the world. Drought is a major factor limiting crop plant production in the world, especially for those regions where rice is mainly cultivated. Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world and it provides staple food for more than half of the world's population. Rice production is directly related to water availability for cultivation. Recently, water deficit has seriously threatened agricultural production and affected food security in all over the world, it becomes dramatically urgent to develop drought tolerant crops. It is expected that collecting information of drought-tolerant cultivars all over the world to understand the molecular basis of desiccation tolerance may help to improve the tolerance of sensitive plants particularly crops to increase food production under water deficit conditions. Plants survive against stress by responding at the cellular, molecular, and organismal levels. On the other hand, response and adaptation of plants to the abiotic stress requires a number of physiological, biochemical, and molecular changes which are accomplished via the expression of several stress-regulated genes (Chinnusamy et al., 2010; Nakashima et al., 2009). Gene expression is controlled by transcription factors that play crucial regulatory roles in approximately every aspect of life in plants, such as plant growth, development, and responses to biotic and abiotic stress (Xiang et al., 2008). The transcription factors play their crucial roles by regulating expression of downstream genes as trans-acting factors via specific binding to cis-acting elements (short nucleotide sequences in the promoter regions of regulated genes) in the promoters of target genes (Mizoi et al., 2012). Specific transcription factors such as members of APETELA2 (AP2), bZIP, zinc finger, NAC, and MYB families have staple roles in regulation of expression of stress-related genes (Oh et al., 2009). Previous studies demonstrated that overexpressing genes encoding specific transcription factors in transgenic plants improve their

tolerance to abiotic stresses such as salinity, cold, and drought (Dubouzet et al., 2003; Vannini et al., 2004; Nakashima et al., 2007; Xiang et al., 2008; Song et al., 2011). bHLH transcription factors have been found to play important roles in phytohormone signaling pathways. bHLH domain and leucine zipper (Leu-Zip) domain are two functionally distinct regions of these proteins (Seo et al., 2011). It is demonstrated that the genomes of rice contain 167 members of the *OsbHLH* family (Li et al., 2006). Additional studies showed that some of these proteins are related to stress responses. For instance, *OsbHLH1* is a cold stress-related gene (Wang et al., 2003).

The bZIP is one of the largest families of transcription factors in plants, several members of this family have important roles in plant stress-responsive and hormone signal transduction (Uno et al., 2000; Jakoby et al., 2002; Rodriguez-Uribe and O'Connell, 2006). A group of bZIP transcription factors family have been found to play essential roles in the abscisic acid (ABA) signaling pathway of *Arabidopsis* plants. However, the understanding about their orthologs's function in rice is still largely limited. The results of phylogenetic analysis revealed that this group is evolutionarily conserved between *Arabidopsis* and rice (Guojun Lu et al., 2009). It is predicted that the indica and japonica genomes of rice contain 88 and 109 genes of bZIP family, respectively (Guo et al., 2005).

The MYB transcription factors contribute to control of the cell cycle and they have been identified, for the first time as oncogenes in animals (Ito et al., 2001). Rice genome contain 183 genes of MYB family that they play various roles in developmental processes and defense responses (Chen et al., 2006). Studies showed that MYB transcription factors are involved in response and adaptation to abiotic stresses (Dai et al., 2007; Ma et al., 2009). For example, *AtMYC2* and *AtMYB2* transcription factors have been revealed to have crucial roles in ABA-dependent gene expression under drought and salt stress (Abe et al., 2003).

The plant-specific NAC transcription factors play important roles in plant development and stress responses. It is predicted that there are 151, and 117 NAC genes in the genomes of rice and *Arabidopsis*, respectively (Nuruzzaman et al., 2010; Pinheiro et al., 2009). Tran et al. (2004) reported that the expressions of *ANAC019*, *ANAC055* and *ANAC072* are induced by drought, high salinity, ABA and methyl jasmonic acid (MeJA) in *Arabidopsis*. Giraudat et al. (1994) showed that the ABA participates plant responses to abiotic stresses, such as high salinity, drought, and low temperature. It has been found that ABA plays essential roles in the accumulation of storage proteins and the acquisition of drought tolerance (McCarty. 1995). *TRAB1* is a transcription factor that is required for ABA-regulated gene expression during seed development (Kagaya et al., 2002). *TRAB1* mediates ABA signaling and Interacts with ABA-responsive elements (ABREs) (Tokunori et al., 1999).

In the present study, the effects of drought stress on expression patterns of five rice genes encoding transcription factors *OsbHLH148*, *OsbZIP72*, *OsMYB2*, *OsNAC6* and *TRAB1* at vegetative and reproductive stages of two Iranian local rice cultivars (Hashemi and Neda) were investigated. These genes are all stress-inducible and may cause improved drought tolerance and have not been previously examined in these two Iranian local rice cultivars. Our objective was to characterize the expression pattern of these genes for the first time in these particular rice cultivars. The result suggests the possibility of using these genes as candidates for developing more varieties of local transgenic rice with improved drought stress tolerance in the future.

Results

Oryza sativa basic helix-loop-helix 148 (OsbHLH148) expression pattern

We studied the effect of drought stress on the gene encoding *OsbHLH148* transcription factor in Hashemi and Neda cultivars at vegetative and reproductive stages. The result revealed a significant increase (7-fold) in expression of *OsbHLH148* in the Hashemi (drought-sensitive) cultivar under drought stress in comparison to control at the reproductive stage. Although the expression of this gene was increased in the Neda (drought-tolerant) cultivar at the vegetative stage, this increase was not statistically significant. Moreover, the expression of *OsbHLH148* was increased in the Hashemi cultivar at the vegetative and reproductive stages, but these increases were not significant (Fig. 1a). As mentioned above, the only significant increase was observed in the Neda cultivar at the reproductive stage, so it shows the importance of this gene's role at reproductive stage in a drought tolerant cultivar.

Oryza sativa basic leucine zipper 72 (OsbZIP72) expression pattern

The qPCR gene expression results as processed and interpreted using REST software indicated that there was a significant increase (4.1-fold) in *OsbZIP72* expression in the Hashemi cultivar under drought stress conditions in comparison to control group at the vegetative stage. As well, in the Hashemi cultivar, the expression of this gene was increased at the reproductive stage, but this increase was not significant. In the Neda cultivar, a modest increase at the vegetative stage was observed, but this increase was not found to be significant. However, *OsbZIP72* expression was risen significantly (3.1-fold) under drought stress conditions in comparison to control at the reproductive stage (Fig. 1b).

Oryza sativa myeloblastosis 2 (OsMYB2) expression pattern

The effect of drought stress on expression of the gene coding for *OsMYB2* is shown in Fig. 1c. The expression results demonstrated that there was a significant increase (3.2-fold) in *OsMYB2* expression in Hashemi at the vegetative stage, as well, the expression of this gene at the reproductive stage was higher than it at the vegetative stage (5.7-fold), but this change was not statistically significant. In the Neda cultivar, the expression of this gene was increased slightly at the vegetative stage, but this increase was not found to be statistically significant. Additionally, the results revealed that there was a sharp increase (8.5-fold) in *OsMYB2* expression at the reproductive stage and this change was statistically significant. Actually, this increase was the highest expression which was observed form our data.

Oryza sativa nam, ataf, and cuc 6 (OsNAC6) expression pattern

The effect of drought stress on expression of the gene coding for *OsNAC6* is shown in Fig. 1d. The qPCR results indicated a significant increase (4-fold) in *OsNAC6* expression in Hashemi (as a drought-sensitive cultivar) in response to drought stress in comparison to control at the vegetative stage. Moreover, the expression of this gene (3.99-fold) in the

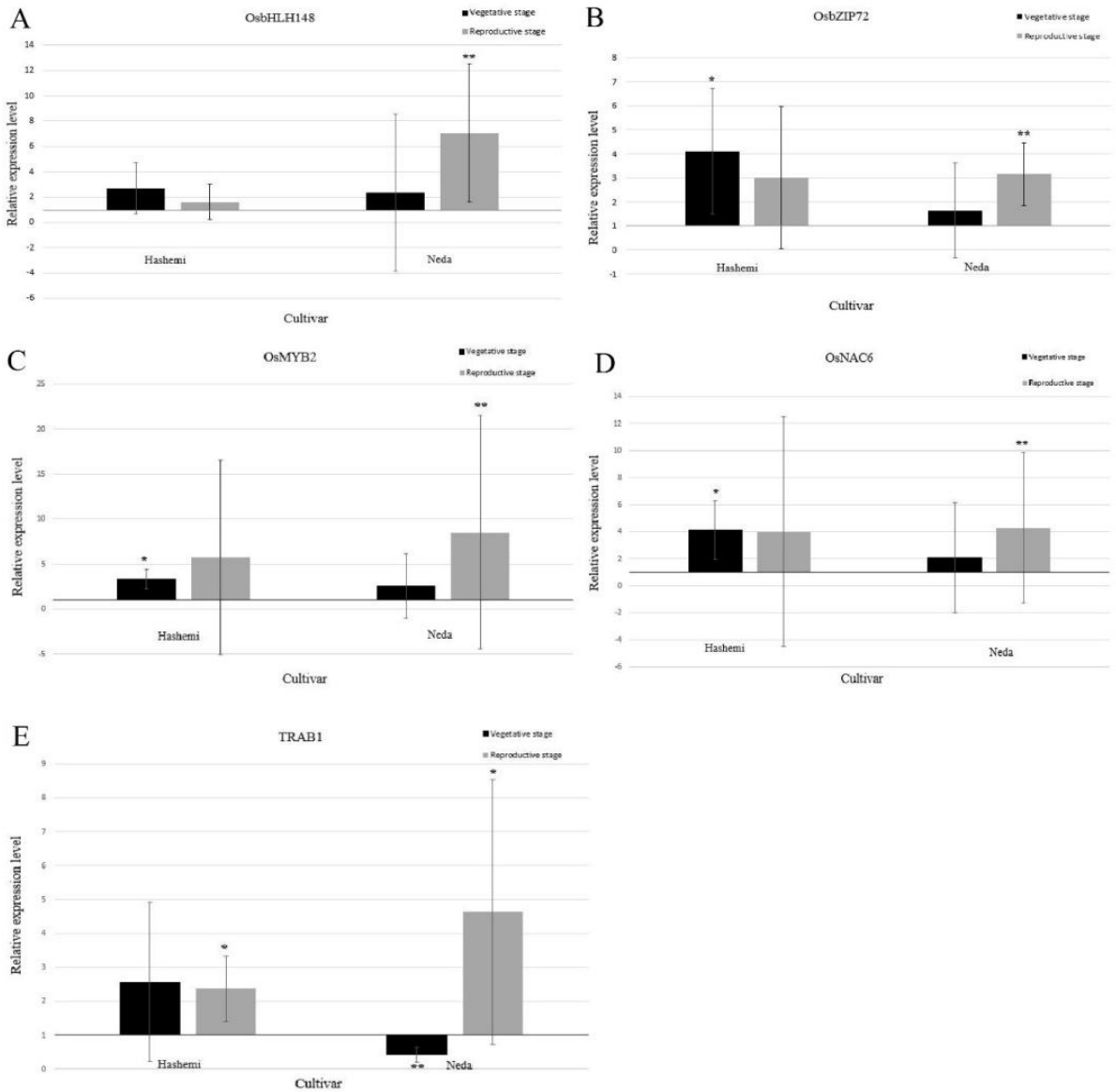

Fig 1. Quantitative PCR (qPCR) was performed for each target gene on three biological replicates for each treatment (stress and control) for each genotype (Hashemi and Neda). Three technical replicates were run for every biological replicate. The qPCR reactions were normalized to 18S rRNA as a reference gene for all comparisons. The Pfaffl method of relative gene quantification was used to make the various comparisons of relative gene expression from the qPCR data, using REST. (A) The expression pattern of OsbHLH148 in Hashemi and Neda cultivars at vegetative and reproductive stages. (B) The expression pattern of *OsbZIP72* in Hashemi and Neda cultivars at vegetative and reproductive stages. (C) The expression pattern of *OsMYB2* in Hashemi and Neda cultivars at vegetative and reproductive stages. (D) The expression pattern of the *OsNAC6* in Hashemi and Neda cultivars at vegetative and reproductive stages. (E) The expression pattern of the *TRAB1* in Hashemi and Neda cultivars at vegetative and reproductive stages.

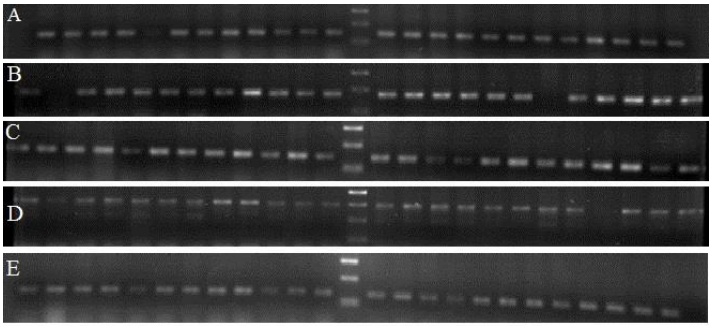

Fig 2. 2%agarose gel electrophoresis. (A) All cDNA samples were tested with *OsbHLH148* primers. (B) All cDNA samples were tested with *OsbZIP72* primers. (C) All cDNA samples were tested with *OsMYB2* primers. (D) All cDNA samples were tested with *OsNAC6* primers. (E) All cDNA samples were tested with *TRAB1* primers.

Hashemi cultivar at the reproductive stage was approximately the same as its expression at the vegetative, but this similarity was not found to be significant. There was a non-significant increase in the expression of this gene in the Neda cultivar at the vegetative stage. The expression data also indicated that there was a significant increase (5.5-fold) in *OsNAC6* expression in the Neda cultivar at the reproductive stage.

Transcription factor responsible for ABA regulation 1 (TRAB1) expression pattern

The expression of gene coding for *TRAB1* in the Hashemi cultivar at the vegetative stage was increased non-significantly, but this gene expression (2.3-fold) was increased significantly at the reproductive stage. In the Neda cultivar, the expression of *TRAB1* was decreased significantly (0.42-fold) at the vegetative stage that this was our only reduction among genes expression data. In contrast, *TRAB1* expression increased significantly (4.6-fold) in Neda under drought stress in comparison to control group at the reproductive stage (Fig. 1e).

Discussion

Gene expression studies in specific cultivars with different responses to drought stress are often the necessary first step to obtain new insights into cultivar-specific mechanisms. Investigation of gene expression patterns of transcription factors family in rice can reveal consequently a better understanding of the molecular mechanisms of these TFs which are involved in acquisition of drought tolerance. Therefore, we investigated the changes of mRNA of five transcription factors under drought stress conditions in two Iranian rice cultivars with drought-sensitive and tolerant backgrounds at the vegetative and reproductive stage.

The reproductive stage is the most sensitive stage to drought stress and if the water deficit occurs during the flowering, there is no way to recover (Yoshida, 1981). As we have herein demonstrated, the drought stress influenced the most in expression of *OsbHLH148* gene in the Neda cultivar at the reproductive stage (Fig. 1a). Neda is a drought tolerant cultivar and this increase in transcript level of the *OsbHLH148* can indicate the importance of this gene's role in tolerance of this cultivar to drought stress. Wang et al. (2003) reported that the *OsbHLH148* was induced by MeJA and ABA or abiotic stresses. In addition, Rice plants with over-expression of the *OsbHLH148* gene demonstrated significantly enhanced drought tolerance. Seo et al. (2011) showed that the production of jasmonate may be increased firstly by drought stress, followed by an increase in ABA production. Consequently the synergistic effect of MeJA and ABA in expression of *OsbHLH148* can enhance the tolerance of transgenic rice to drought stress.

According to the expression analysis using quantitative RT-PCR abiotic stresses, ABA and ACC (ethylene) significantly induced the expressions of most of *OsbZIP*s genes family (Guojun Lu et al., 2009). Guojun Lu et al. (2009) demonstrated that over-expression of *OsbZIP72* caused hypersensitivity of transgenic rice to ABA which enhanced expression of ABA response gene such as late embryogenesis abundant proteins (LEAs), and improved drought tolerance, Therefore, they suggest that *OsbZIP72* plays a positive role in drought resistance through ABA signaling. In addition, *OsbZIP72* is the first positive regulator which identified in this group of transcription factors (Guojun Lu et al., 2009). Based on our results, the expression of *OsbZIP72* was increased significantly in Hashemi as a drought sensitive cultivar at the vegetative stage but it showed no significant change at the reproductive stage as the most sensitive stage to drought stress. However, our results revealed that there was a significant increase in expression of *OsbZIP72* in Neda as a drought tolerant cultivar at the reproductive stage. As shown in Fig. 1b, this gene only acted at the vegetative and reproductive stages in Hashemi and Neda cultivars, respectively. It seems, *OsbZIP72* is participated in drought tolerance of Neda cultivar at the reproductive stage through ABA signaling.

Kranz et al. (1998) reported that MYB proteins are involved in the regulation of secondary metabolism, control of cellular morphogenesis, and regulation of the meristem and the cell cycle of *Arabidopsis*. Yang et al. (2012) demonstrated that plants with over-expression of the *OsMYB2* gene showed a higher survival rate than wild-type or RNAi plants to multiple abiotic stresses such as salt, cold, and dehydration stress. Moreover, the expression of *OsMYB2* was increased by exogenous ABA, and transgenic plants with over-expression of the *OsMYB2* were more sensitive to exogenous ABA than the wild type in terms of seed germination and seedling growth (Yang et al., 2012). In contrast, the expression of *OsMYBS3* in leaves were suppressed by exogenous ABA, while its expression in roots did not change to exogenous application of ABA (Su et al., 2010). According to our results, the expression of *OsMYB2* was increased in Hashemi at the vegetative stage. Based on previous studies and *OsMYB2* roles, it seems that this gene plays a positive role in Hashemi cultivar with drought-sensitive backgrounds at the vegetative stage. However it did not show any positive role at the reproductive stage as the most critical stage to drought stress in this cultivar (Fig. 1c). This may indicate an important role of this gene in the beginning of a plant's life. In addition, we have shown that drought stress caused significant increase in expression of *OsMYB2* in Neda cultivar that has drought tolerant background at the reproductive stage. Therefore, this gene might play an important role in drought tolerance of this cultivar at the reproductive stage through accumulation of osmolytes. Yang et al. (2012) reported that the rice plants with Overexpression of *OsMYB2* showed higher accumulation of compatible osmolytes, such as soluble sugars, free proline, and LEA proteins under abiotic stresses.
Molecular characterization of the eight *OsNAC* genes, *OsNAC1* to *OsNAC8* have been performed, these genes encode proteins with NAC domains in rice (Kikuchi et al., 2000). The *OsNAC6* has been reported as one the most important member of the NAC proteins in rice. Nakashima et al. (2007) showed that the expression of *OsNAC6* was induced by cold, drought and high salinity stresses, and rice plants with over-expression of *OsNAC6* showed tolerance to dehydration and high-salt stresses. Moreover, *OsNAC6* gene expression was also induced by wounding and blast disease (Nakashima et al., 2007). A transient transactivation assay revealed that *OsNAC6* activates the expression of at least two genes, including a gene encoding peroxidase enzyme (Nakashima et al., 2007). Based on our results, a significant increase in expression of *OsNAC6* was observed in Hashemi cultivar at the vegetative stage. However, there was no any significant change in expression of this gene in Hashemi as a sensitive cultivar to drought stress at the reproductive stage. On the other hand, the expression of *OsNAC6* showed no significant change in Neda cultivar at the vegetative stage, but it was increased significantly in this cultivar as a drought tolerance cultivar at the reproductive stage as the most sensitive stage to drought stress (Fig. 1d). It seems that the *OsNAC6* might be involved in the tolerance of Neda cultivar

to drought stress at the reproductive stage and it might play essential role in tolerance of this cultivar in response to drought stress.

Hobo et al. (1999) characterized a basic region leucine zipper (bZIP) factor, *TRAB1* that interacts with both VP1 and ABA-responsive elements (ABREs). Maturation and dormancy in plant seeds are regulated by VP1 as a transcription factor through activating genes responsive to the stress hormone ABA. This activation involves ABA-responsive elements, and *TRAB1* interacts with both VP1 and ABREs. Hobo et al. (1999) demonstrated that VP1 acts through interaction with a factor named *TRAB1* that directly binds to ABRE, thereby regulating ABA-induced transcription. Hobo et al. (1999) reported that the northern analysis revealed that the expression of TRAB1 was increased by exogenous ABA. As shown in Fig. 1e, we observed a significant increase in expression of *TRAB1* in Hashemi cultivar with drought sensitive background at the reproductive stage. *TRAB1* is the only gene among our genes of interest which showed increase in this cultivar at the reproductive stage as the most sensitive stage to drought stress. In Neda as a drought tolerant cultivar, there was a significant decrease in expression of *TRAB1* at the vegetative stage. This is the only significant reduction among the genes of interest which was observed at the vegetative stage. It seems *TRAB1* is sensitive to drought stress in Neda cultivar at the vegetative or tillering stage. However, a significant increase in *TRAB1* expression in Neda at the reproductive stage was observed. The increased expression of this gene in the Neda cultivar at this stage may contribute to the tolerance it demonstrates to drought stress at the reproductive stage.

Materials and Methods

Plant growth and drought-treatment conditions

Seeds of two local cultivars (Hashemi and Neda) of rice (*Oryza sativa* L. sub. *indica*) were obtained from the Rice Research Institute of Iran (RRII). Based on the drought indices results in previous study, the Hashemi cultivar is sensitive to drought stress while the Neda cultivar is drought tolerant (Mollazadeh Taghipour et al., unpublished). The seeds were sterilized, germinated, planted in seedling boxes in a greenhouse, and then 19- day-old seedlings were transferred to larger pots. The pots were covered with plastic to avoid water wasting. Before the application of drought stress, the pots were watered once daily to keep the soil saturated. The drought stress was applied by stopping irrigation at the tillering stage (30-day-old plants) for 18 days and heading stage (70-day-old plants) for 14 days for Hashemi cultivar and 19 days for Neda cultivar. The Neda cultivar in comparison to Hashemi started later to show visual symptoms of drought-induced damages. As well, Plants maintained under optimum irrigation (untreated plants) were used as experimental controls. Leaves were sampled after observation of drought-induced symptoms; such as leaf rolling and wilting with a concomitant loss of chlorophylls, and stored at -80˚C.

Gene expression analysis

The gene sequences for *OsbHLH148* (accession no. DI218613), *OsbZIP72* (accession no. AK065873), *OsMYB2* (accession no. AK120551), *OsNAC6* (accession no. AB028185) and *TRAB1* (accession no.AB023288) were obtained from the NCBI database. Gene-specific primers were designed using the online software, Primer 3, for all five genes. *18S-rRNA* (Huang et al., 2012) was used as the single reference (housekeeping) gene for normalization of the quantitative PCR target data (Table 1).

RNA extraction

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions (with some modifications) from drought stress treated and untreated plants at the vegetative and reproductive stages. The quality and quantity of RNA samples were assayed by 1% Agarose gel electrophoresis and NanoDrop (NanoDrop2000), respectively.

cDNA synthesis

First-strand cDNA was synthesized according to manufacturer's instructions (with some modifications) using2 µg of total DNase-treated RNA per reaction in a M-MuLV reverse transcription system. The master mix contained 2.5 µl RNA, 11.25 µl nuclease free water, 5 µl 5X reaction buffer, 1.25 µl RiboLock RTRNase inhibitor (20 units/µl), 2.5 µl 10 mMdNTP mixture, 1.25 µl random hexamer primers (0.2 µg/ µl) and 1.25 µl M-MuLV reverse transcriptase (200 units/µl) in 25 µl final reaction volume. The volume of the RNA and nuclease free water were adjusted according to the RNA concentration in different samples so that all reverse transcription reactions contained the same amount of RNA. PCR gradient was used to determine the annealing temperature of each set of primers. The quality of all cDNA samples was examined with all sets of primers, and PCR products were tested using 2% agarose gel electrophoresis (Fig. 2).

Quantitative PCR

Real-time quantitative PCR (qPCR) was performed in a manner consistent and compliant with the MIQE Guidelines (Bustin et al., 2009) in an optical 96-well plate with a BIO-RAD iQ5 real-time PCR system (BIO-RAD, USA); three technical replicates were used for all sample and standard assessments. Thermo-cycling conditions were: 95ºC for 4 min, followed by 45 cycles at 95ºC for 30 s, 61ºC for 30 s, and 72ºC for 30 s, with a melting curve of 81 cycles at 55ºC to 95ºC for 10 s. Each single-plex reaction contained 7.5 µl SYBR Green I/ROX qPCR master mix (Invitrogen), 1 µl of each forward and reverse primer (final concentration of 10000 nM each), 3.5 µl nuclease-free water, and 2 µl cDNA at an optimal concentration as determined by the Stock I/PREXCEL-Q Method for qPCR (Gallup and Ackermann, 2006, 2008). In this experiment, the Pfaffl model was used for data processing (Pfaffl, 2001). This method combines gene quantification and normalization into a single calculation and incorporates the amplification efficiencies of the target and reference (normalization) genes, and avoids the erroneous assumption of 100% amplification efficiencies for all targets that the $2^{-\Delta\Delta Ct}$ method is based on. The relative expression software tool (REST©), which runs in Microsoft Windows XP, automates data analysis using this model (Pfaffl et al., 2002). REST uses the Pairwise Fixed Reallocation Randomization Test© to calculate result significance and indicates if the reference gene(s) used is/are suitable for normalization (Marisa et al., 2005). Equation (1) shows the simplest form of the Pfaffl mathematical model which includes an efficiency correction for real-time PCR efficiency for both target and reference genes.

Ratio (fold change) = $(E_{target})^{\Delta Cqtarget(control - sample)}/(E_{reference})^{\Delta Cqref(control - sample)}$ (1)

The exponential amplification efficiency (E) of a reaction was calculated (using data collected from a standard curve for each of 5 genes) by the following formula (2). To obtain standard curves that were designed within the optimal dynamic dilution range for each target, the Stock I/PREXCEL-Q Method for qPCR was employed.

$E=10^{(-1/slope)}$ (2)

Representative sample mixture (Stock I)-derived standard curves demonstrated amplification efficiencies of 99.2%, 98.3%, 93.6%, 94.6%, 99.2%, 81.2% for *OsbHLH148*, *OsbZIP72*, *OsMYB2*, *OsNAC6*, *TRAB1* and *18S-rRNA*, respectively. Standard curve efficiencies were calculated by the following equation (3):

$E_{stnd\ curve} = [10^{(-1/slope)}] - 1$ (3)

Statistical analysis

The experiment was carried out by using a 3 factorial experiment in a completely randomized design (CRD) with three biological replicates. Factor A was genotype with 2 levels (Hashemi and Neda cultivars), factor B was drought stress with 2 levels (stress and non-stress), and factor C was the growth stage to which stress was applied, with 2 levels (vegetative and reproductive). Data were analyzed using the REST software.

Conclusion

The reproductive stage is the most sensitive stage to drought stress and if the water deficit occurs during the flowering, there is no way to recover. Neda genotype showed significant increase in expression of transcript level of all five genes *OsbHLH148*, *OsbZIP72*, *OsMYB2*, *OsNAC6* and *TRAB1* at the reproductive stage. It indicates the importance of these five drought stress responsive genes in acquisition of drought tolerance in this genotype. However, there were no significant changes in expression of *OsbHLH148*, *OsbZIP72*, *OsMYB2* and *OsNAC6* but a significant decrease in expression of *TRAB1* in Neda cultivar at the vegetative stage. It seems, these genes play their roles only at the reproductive stage in Neda genotype. On the other hand, Hashemi genotype did not show any significant changes in expression of *OsbHLH148*, *OsbZIP72*, *OsMYB2* and *OsNAC6* but only a significant increase in expression of *TRAB1* at the reproductive stage. Hashemi also showed significant increase in expression of *OsbZIP72*, *OsMYB2* and *OsNAC6* at the vegetative stage. Therefore, these three genes are drought responsive in Hashemi at the vegetative stage.

Conflict of interest

The authors declare that they have no conflict of interest.

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