OVEREXPRESSION OF *OsHox*-6 GENE ENHANCED TILLER NUMBER IN RICE BUT INDUCED YIELD PENALTY

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Abstract

OsHox-6, belongs to the transcription factor homeodomain leucine zipper (HD-Zip) protein sub-family I, has unknown function. This study was aimed to characterize the phenotypes of two homozygous transgenic rice lines (S29-62-2 and S.40.4-158-1) containing an extra copy of *OsHox*-6 gene under the control of a rice constitutive promoter, OsLEA3, and to evaluate their tolerance to water stress. A real-time quantitative PCR (qRT-PCR) showed that the transcript expression of *OsHox*-6 gene in the transgenic lines increased 5-10 folds under a normal irrigation and 10-20 folds after exposure to water stress conditions as compared to its wild type control. Transgenic plants overexpressing *OsHox*-6 exhibited phenotypic alteration at the normal irrigation by inducing tiller formation, suggesting a decrease in the apical dominance. Transgenic plants also showed significant enhancement in the total grain number, however, the number of empty grains also increased significantly (~16-22%). After imposed to the water stress, the number of empty grains in the transgenic lines was even higher (up to 83% in average). Furthermore, observations on the water loss rates, relative water contents and drought resistance indices (DRI) suggested that the overexpression of *OsHox*-6 did not significantly increase tolerance to water stress. Further research is required to reveal the detailed mechanisms of *OsHox*-6 in response to water and other abiotic stresses.

Keywords: rice, drought, IR64 Sub1, HD-Zip, *OsHox*-6

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Introduction

Homeodomain leucine zipper (HD-Zip) is one of the transcription factors found uniquely in plants, containing an essential homeodomain region for DNA binding and a leucin zipper motif that helps protein dimerization. The protein dimerization is required to allow the DNA binding processes to take place. This HD-Zip transcription factors are a large family which are divided into IV sub-families based on the homeodomain DNA binding specificity, the structure and function of genes, and the presence of other motives (Ariel *et al*., 2007).

Previous studies on different plant species showed that the HD-Zip genes have highly diverse functions. Some of the well-known HD-Zip proteins are GLABRA, PHABULOSA, and REVOLUTA. GLABRA belong to the HD-zip sub-famili IV, while PHABULOSA, CORONA and REVOLUTA are members of HD-Zip sub-families III. The GLABRA genes are important in the formation of trichomes (Rerie *et al*., 1994), whereas the REVOLUTA, PHABULOSA and CORONA genes play important roles in the development of leaves and vascular tissues, and in the initiation of meristems (Prigge *et al*., 2005).

The HD-Zip genes, particularly sub-families I and II, have been of particular interest of researchers, especially for their role in the mechanism of drought tolerance in plants. Five HD-Zip genes (*CpHB-3, -4, -5, -6, -7*) of *Craterostigma plantagineum*, a desert and extremely drought tolerant plant, were found to be regulated during dehydration that lead to a desiccation tolerance (Deng *et al*., 2002). Previously, Frank *et al*. (1998) isolated 2 HD-Zip genes (*CpHB-1* & *-2*) of *C. plantagineum* which were responsive to dehydration at the early stages of the treatment, however, no further reports on the functions or any tolerance mechanisms related to these HD-Zip genes in response to water stress.

A promising report came from Dezar *et al*. (2005) who studied the *Hahb-4* gene (HD-Zip

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sub-families I) of the sunflower (*Helianthus annuus*). Overexpression of *Hahb-4* driven by CaMV35S promoter in *Arabidopsis thaliana* increased its tolerance to water stress as indicated by higher survival rates compared to the untransformed parental lines. This report suggested the potential use of HD-Zip genes in developing drought-tolerant plants.

In 2007, Ariel *et al*. has isolated 26 HD-Zip genes belonging to the sub-families I and II from the model plant *Arabidopsis thaliana*. Four of them, *AtHB-5, -6, -7, and -12*, were regulated during dehydration. Water stress down regulated the expression of *AtHB-5* and *-6* genes, but up regulated the expression of *AtHB-7* and *-12*. Previously, Hjellstrom *et al*. (2003) and Olsson *et al*. (2004) reported that *AtHB-7* and *-12* play an important role in cell elongation. Overexpression of *AtHB-7* and *-12* lead the plants to become shorter due to reduction in their cell length.

Information on the function and regulation mechanisms of HD-Zip genes in rice is still limited. HD-Zip transcription factors in rice plant were just actively studied in recent ten years. An earlier study came from Agalou *et al*. (2008) who found as many as 31 of HD-Zip genes in rice that belong to the sub-families I, II, and III. Agalou *et al*. reported that 8 out of 31 rice HD-zip genes studied, i.e *OsHox*-4, -6, -11, -19, -20, -22, -24, and -27 that belong to the sub-family I and II, were regulated during dehydration. *OsHox*-4 genes played a role in the elongation and enlargement of the vessel cells (Agalou *et al*. 2008). Overexpression of *OsHox*-4 caused a semi-dwarf phenotype and significant reduction in the internode length and the tiller number (Agalou *et al*. 2008; Dai *et al*. 2008). *OsHox*-22 was predicted to play an important role in the ABA biosynthesis. Overexpression of *OsHox*-22 increased sensitivity to ABA and dehydration. Down regulation of rice *OsHox*-22, however, significantly increased rice drought tolerance at the seedling stage (Zhang *et al*. 2012). Similar results also came from Bhattacharjee *et al*. (2016) who worked on *OsHox*-22 and -24. Overexpression of *OsHox*-22 and -24 in *A. thaliana* caused greater plant sensitivity to ABA, dehydration and salt stresses. Although HD-Zip transcription factors have been studied in the past two decades, their functions and mechanisms of action in plants, especially in response to water stress, remain unclear. Further studies on the other members of rice HD-zip genes are also required to gain more comprehensive understanding on the roles of HD-zip genes in drought stress. The findings will be very important to reveal the potential of these genes as candidates for the development of drought tolerant crops.

In the present work, the function of *OsHox*-6 gene in response to water stress was studied. A previous study showed that the transcript levels of *OsHox*-6 gene were increased during water stress conditions, accumulated in rice nodes, roots and pedicels (Agalou *et al*. 2008; Rahmawati, 2012). Previously, we have transformed rice cv IR64 Sub1 with pCAMBIA1301H carrying the *OsHox*-6 gene under the control of OsLEA3 as a drought-inducible promoter and resulted several homozygous transgenic lines. OsLEA3 was known to perform high activity in water stress conditions (Xiao *et al*., 2007). We then selected two homozygous lines overexpressing *OsHox*-6 for further analyses. The phenotypes of the transgenic lines overexpressing *OsHox*-6 and their tolerance to water stress were evaluated.

Materials and Methods

Plant Materials

Two homozygous transgenic lines (S29-62-2, and S.40.4-158-1) of rice (*Oryza sativa* L. ssp indica cv IR64 Sub1) carrying the *OsHox*-6 gene under the control of OsLEA3 promoter were used. The untransformed rice IR64 Sub1 was used as a control in all experiments.

qRT-PCR

Total RNA was isolated from the young leaves of transgenic and non-transgenic plants before and after air-dried for 1 hour at a room temperature in the lab. Total RNA was extracted from leaves using Trizol LS® Reagent (Invitrogen), following the manufacturer's protocol. Before extraction, total RNA samples were treated with RNase-free DNase I (Thermo Scientific) to remove DNA contaminants. For the quantitative Real Time PCR, KAPA SYBR FAST® one-step qRT-PCR kit (Invitrogen) was used. The qRT-PCR reaction mixture contained 5 µl KAPA SYBR FAST buffer, 0.2 µl each 10 µM forward and reverse primers, 0.2 µl 10 µM dUTP, 0.2 µl KAPA RT-mix, and 40 ng RNA

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sample, in a total volume of 10 µl. Two pairs of primers (Table 1) were used to amplify *OsHox*6 and *actin* transcripts, respectively, according to Agalou *et al*. (2008). Each sample was prepared in duplicates. Template RNA was initially reverse transcribed into cDNA at 42°C for 5 minutes. The cDNA was then denatured at 95°C for 5 minutes, followed by 40 cycles of PCR amplification in the following conditions : a 3-s denaturation at 95°C, a 30-s annealing at 60°C, and a 30-s primer extension at 72°C to allow completion of primer extension. Quantitative RT-PCR results were analyzed using the software provided by the Eco™ Illumina instrument.

Table 1. The sequences of primers used in the qRT-PCR analysis of *OsHox*-6 expression

No.	Primer	Sequence 5' → 3'
1.	OsActin fwd	CTG GGT TCG CCG GAG ATG AT
2.	OsActin rev	TGA GAT CAC GCC CAG CAA GG
3.	Oshox6rtF	GGC CGT CGT CCA CGG A
4.	Oshox6rtR	TCG CTC TCG AAT TCC CAC C

Morphological Characterization of Transgenic Lines

Wild type (WT) and transgenic rice lines were grown in pots, one plant per pot in five biological replicates. All plants were grown in standard irrigation conditions. Fertilizers were applied as recommended. Pest and disease control were carried out when necessary. Observation on agronomic characteristics, i.e. plant height, tiller number, number of productive tillers, flowering time (the time when the number of flowering plants are more than 50%), total grain number, filled grain number, and weight of 1000 seeds were performed for the mature plants at the reproductive stage.

Quantification of Water-loss at the Seedling Stage

Quantification of water loss was carried out according to Yu *et al*. (2008). The shoots of 21-day old WT and transgenic seedlings were detached and weighed immediately (FW_0). The leaves were then incubated in Petri dishes at a room temperature. The fresh weight (FW) was measured at 10 min interval for two hours. The rates of water loss were calculated from the equation:

Water loss (%) = $(FW_0\text{-}FW)/(FW_0) \times 100\%$.

Drought Resistance Test at the Generative Stage

Plants were grown in PVC pots (ϕ 20 cm and height 80 cm) during a dry season (April-August 2012) in a green house. Each pot was filled with 16 kg of soil, sand and cocopeat (2:1:1). Three seedlings were transplanted into each pot and after three weeks only one seedling which grew best was left per pot. Plants were fertilized with 0.4 g TSP and 0.3 g KCl a day after transplanting while 0.3 g N was added at the week 1, 4, and 6 after transplanting. All the plants were grown normally until the time of imposing the water treatment (9 weeks after transplanting). The plants were then divided into two sets of treatments with three replicates of each line. The dry down experiment was done according to Kholova *et al*. (2010). A day before the water-stress treatment, the soil was saturated with water and allowed to drain overnight (day 0). In the morning of the following day, the upper pots were covered with transparent plastic bags that wrap around the pots and the stems. The pots were weighed in every morning during the first three days, and after that, the pots were weighed every two days. One set of the treatment was maintained under a well-watered (WW) condition by daily re-watering until the initial weight was reached. Another set was gradually exposed to water stress (WS) by partially compensating water loss from transpiration (plants were allowed to lose no more than 200 g each day). The experiment was terminated when the transpiration of WS plants <10% of WW plants. The duration of the water stress experiment was 43 days. Plants were then rehydrated, and at harvest time, agronomic characteristic (i.e. plant height, tiller number, number of productive tillers), biomass, and yield were recorded. After the harvest, the fraction of transpirable soil water (FTSW) for each day was calculated using the equation:

FTSW = (pot weight of day n-final pot weight)/ (initial pot weight-final pot weight).

The tolerance levels of transgenic plants were evaluated by looking at the resistance index (Drought Resistance Index, DRI) using the equation: DRI = (Ys / Yn) / (Ms / Mn). Ys and Yn are plant yield or biomass under stress

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(WS) and normal (WW) condition respectively, while Ms and Mn are the average yield or biomass across the genotypes tested consecutively under stress and normal conditions (Fischer & Maurer, 1978).

Quantification of Relative Water Content (RWC)

RWC of the leaves was measured at day-0 and day-28 after exposure to water stress treatments. Fully expanded leaves were cut from plants and their fresh weight (FW) were recorded immediately. The leaves were then immersed in distilled water for 16 h and the turgid weight (TW) values were recorded. The leaves were further dried in an oven for 72 h at 80°C and the dry weight (DW) values were recorded. The RWC values were calculated by using the equation: RWC (%) = (FW-DW)/(TW-DW) x 100%.

Statistical Analysis

Agronomic characteristics and drought resistance index of the transgenic lines and WT were analyzed for their variation with respect to the effects of genotypes and treatments using the analysis of variance (ANOVA) by the SAS software version 8.0. The statistical significance of differences was analyzed based on the Fisher's LSD test.

Results

Overexpression of *OsHox*-6 Gene in The Transgenic Rice Plants

Real-time quantitative PCR (qRT-PCR) was performed to measure the expression of *OsHox*-6 in the leaves of the transgenic rice lines during water stress. The result showed that the expression of the *OsHox*-6 gene was increased in all the transgenic lines tested. The expression levels of *OsHox*-6 gene in these lines were 5 to 10 times higher than those of untransformed control plants (Figure 1) under a normal condition. When the leaves were imposed to dehydration, the expression of the *OsHox*-6 gene was even higher 10 to 20 times. The highest expression was found in the transgenic rice line S29-62-2.

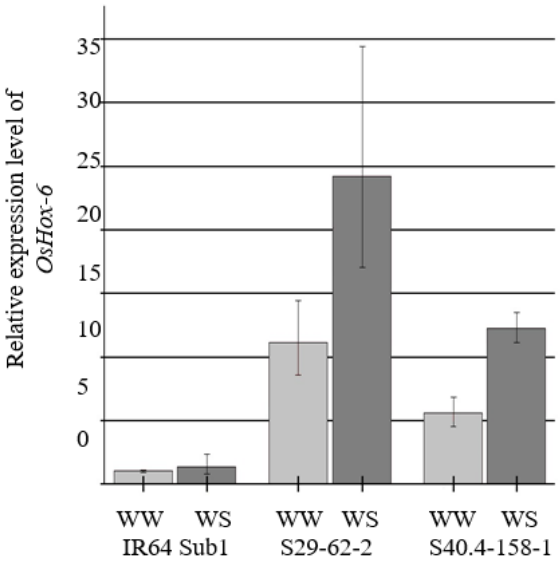

Figure 1. The relative expression of the *OsHox*-6 gene in two transgenic rice lines (S29-62-2 and S.40.4-158-1) revealed by a real-time quantitative PCR analysis. Total RNA was extracted from leaves before/well-watered (WW) and after dehydrated for 1 hour at the room temperature/water stress (WS). Actin was used as a reference gene. Values represent the means of two replications.

Phenotypes of *OsHox*-6 Overexpressed Transgenic Rice Lines

Under a normal irrigation, we observed no significant differences between WT and *OsHox*-6 over-expressed transgenic lines in plant height. Interestingly, the tiller number of both transgenic lines were around 30-60% more than the wild type. Likewise, the number of productive tillers of the transgenic lines were 25-60% more than that of WT. Meanwhile, the total grain number was significantly different between *OsHox*-6 over-expressed transgenic lines and its wild type. Unfortunately, the percentage of empty grains were also increased by 2-3 times in the transgenic lines. We also observed a 2-days delay of the flowering time in both transgenic lines. The phenotypes that are related to the important agronomic parameters are presented in the Table 2.

Overexpression of *OsHox*-6 Gene Did Not Significantly Improve the Water Loss Rate, Relative Water Content and Drought Resistance Index

There are a number of parameters that have been used to evaluate or select drought tolerant plants. Several drought tolerance mechanisms

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are associated with leaf water status such as transpirational water loss and relative water content. Water status of the plants that maintain cell turgor under water stress conditions are often used as the parameter to select drought tolerant or drought-avoidance plants (Hu & Xiong, 2014). One simple and quick method to measure the rate of leaf water loss from plants is by using detached leaf method as described previously by Tang *et al*. (2012) and Yue *et al.* (2012).

Table 2. Agronomic characteristics of transgenic rice lines overexpressing *OsHox*-6 under standard irrigation conditions.

	Lines		
Parameters	IR64 Sub1	S29-62-2	S40.4-158-1
PH (cm)	91,20± 3.054a*	92,90± 4.278a	89,25± 2.062a
TN	12 ± 2.1a	16 ± 4.0ab	20 ± 4.0b
PTN	12 ±2.2a	15 ± 4.1ab	20 ± 4.0b
FT	78	80	80
TG	1178±192.8a	1536±375.9b	1715±336.0b
FG	1093±165.9a	1284±268.1a	1335±288.8a
% EG	7,22 ± 2.623a	16,41 ±4.235b	22,17 ±6.892b
SW1000	22,18 ± 1.140a	21,73 ± 0.546a	21,67 ± 0.423a

Note: PH: plant height, TN: Tiller number, PTN: Productive tiller number, FT: Flowering time (days after transplanting), TG; total grain, FG: filled grain, EG: empty grain, SW1000: weight of 1000 seeds (gram).
* means sharing similar letter in a line do not differ significantly at $\alpha = 0.05$

Using the detached leaf method, we monitored the rates of water loss from detached leaves of the transgenic rice lines overexpressing the *OsHox*6 gene over the time. In the beginning, the rates of water loss of the transgenic lines were slightly below those of the non-transgenic IR64 Sub1 at the seedling stage, but not significantly different. After 2 h exposure to dehydration, the rates of water loss of the transgenic lines were faster than those of the control wild type (Figure 2).

A similar trend was also observed on the relative water content (RWC). The measurement of the RWC was used to determine the hydration status of leaves relative to their maximal water holding capacity at full turgidity. Leaf water status of the transgenic rice lines and the IR64 Sub1 control under the well-watered condition were similar. When exposed to the water stress for 4 weeks, the RWC of the IR64 Sub1 control was decreased to less than 80% while the transgenic rice lines were able to maintain their RWC above 80% (Figure 3).

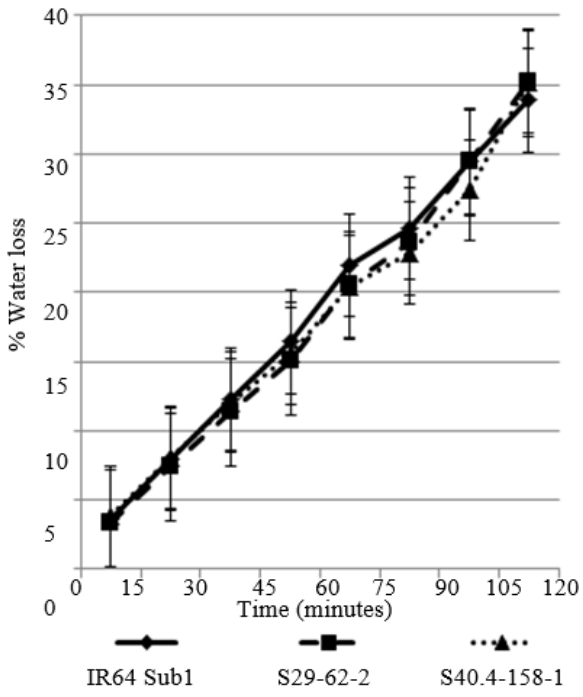

Figure 2. Comparison of water loss rates from detached shoots of the plants at the normal condition. Water loss represents the weight proportion of the water that loss by transpiration as compared to the initial weight. Values represent the means of 3 shoots from one plant.

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The reduction of RWC in the IR64 Sub1 control was, however, not significantly different with those of the transgenic rice lines, indicating that all the lines tested had relatively equal ability to maintain cell turgidity under the water stress condition.

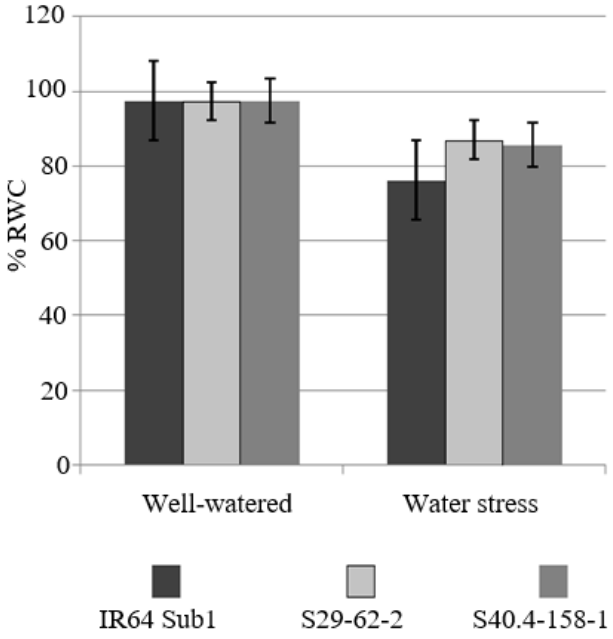

Figure 3. Leaf RWC of transgenic lines and IR64 Sub1 control under well- watered (WW) and water stress (WS) conditions.

Table 3. DRI of transgenic rice lines overexpressing *OsHox*6 and IR64 Sub1 control.

Lines	DRI	
	Biomass	Total Grain
IR 64 Sub 1	0.80 ± 0.189a*	0.68 ± 0.214a
S29-62-2	1.23 ± 0.217a	1.27 ± 0.319a
S40.4-158-1	1.09 ± 0.187a	1.21 ± 0.296a

Note: * means sharing similar letter in a column do not differ significantly at α = 0.05

Another criterion that commonly used to evaluate and select drought tolerant plants under drought condition is drought resistance index (DRI) (Farshadfar *et al*. 2013, Razzaq *et al*. 2013). This criterion has been considered as the best criterion for evaluation of resistant cultivars (Hu *et al*. 2007) and has been used as a standard in the identification of drought tolerant rice and wheat in China (Li *et al*. 2006). Cultivars with greater DRI values are considered more resistant to drought.

The DRI values of the two homozygous lines tested in this experiment were presented in Table 3. The DRI was calculated based on the biomass and total grain (yield). The highest DRI was obtained for line S29-62-2 with DRI value of 1.23 and 1.27 for biomass and total grain, respectively. The DRI values were, however, not significantly different at 5% of probability.

Effects of Drought on Growth Parameters

Results from the observation on the agronomic characteristics of the transgenic lines overexpressing *OsHox*-6 and the wild type are presented in the Table 4. There was no significant difference in plant height between the transgenic lines with the WT before imposing to water stress. Plant height, however, was reduced significantly in transgenics as compared to the WT after the treatment (Figure 4, Table 4) . We also observed that there were no significant differences in the tiller number and the productive tiller number between the transgenic lines and the WT IR64 Sub1 during the normal water condition. Meanwhile, the transgenic lines were able to recover faster than its wild type after the stress treatment by producing more tillers and productive tillers as indicated by the emergence of new panicles.

Observation on the yield parameters showed that under the well-watered conditions, transgenic lines produced total grain number less than the WT control. The total grain number in the transgenic lines tend to increase under the water-stress, whereas in the WT plants tend to decrease. However, under the water stress, there was no significant difference between the total grain number in the transgenic and non-transgenic lines Unfortunately, the percentage numbers of empty grains were also increased in transgenic plants overexpressing *OsHox*-6 grown under the normal and treatment conditions.

In the well-watered condition, the biomasses of rice transgenic lines were lower than that of the IR64 Sub1 control. However, after imposed to the water stress, the biomass of the non-transgenic IR64 Sub1 decreased to 25% while biomass production of the transgenic lines, S40.4-158-1 and S29-62-2, increased 2.5% and 15.2% respectively along with the increases of tiller numbers.

Discussion

Overexpression of *OsHox*6 Gene in The Transgenic Rice Plants Changed Plant Architechture

Homeodomain leucine zipper (HD-Zip) transcription factors have been widely studied in the past few decades since they were found to be involved in various processes of plant growth and development in response to the environmental changes including abiotic (drought, salt, and osmotic stresses) and biotic stresses (virus) (Ariel *et al*. 2007, Ribone *et al.* 2015). HD-Zip transcription factors play a role as growth regulators in response to abiotic stresses including water stress.

Our results on this study also support that HDZip transcription factors, from which the *OsHox*-6 gene is one of the members of the subfamily 1, play a role in regulating plant growth as a response to water stress. Based on our data, the overexpression of *OsHox*-6 transcription factor did not significantly reduced plant height as previously reported on its homolog, including *OsHox*-22, *OsHox*-24, *AtHb*-7, and *AtHb*-12 (Hjellstrom *et al*. 2003, Olsson *et al*. 2004, Zhang *et al*. 2012, Bhattacharjee *et al.* 2016). However, the overexpression of *OsHox*-6 significantly increased tiller number, indicating that it might play an important role in tiller formation. Furthermore, we observed that several seeds germinated with 2-3 shoots (Rahmawati, 2012) where this phenomenon was not found for seeds originated from its WT control.

Similar results were previously reported by Hjellstrom *et al*. (2003) on *Arabidopsis AtHB*-7 transcription factor, one of the homologs of *OsHox*-6 gene which had been well studied earlier. The *Arabidopsis* lines over-expressing *AtHB*-7 have more branches in the main inflorescence stem compared to the wild-type control, suggesting the decrease in apical dominance in the plants.

Tiller number and productive tiller number are two agronomic characteristics that are important in crop breeding to improve plant biomass and yield. Thus, finding the key genes in tiller formation would be very important to improve crops productivity (Zhang *et al*. 2009). Several genes involved in tiller formation have been found and characterized, including *OsNPF*-7.2 (Wang *et al*. 2018), *OsMax*-1 (Wang *et al*. 2015), and some other genes as described by Hussien *et al*. (2014). These genes may have interactions with or become the target of the *OsHox*-6 transcription factor. Thus, further research is still needed to discover the target genes of this transcription factor.

Table 4. Phenotypes of transgenic lines over-expressing *OsHox*-6 after exposed to water stress conditions.

Parameters		Lines		
		IR 64 Sub1	S29-62-2	S40.4-158-1
PH (cm)	WW	95.93 ± 1.401a*	97.17 ± 2.754a	90.83 ± 5.252a
	WS	89.83 ± 2.485a	87.43 ± 2.542ab	81.27 ± 3.301b
TN	WW	15 ± 2.3a	14 ± 1.2a	15±7.4a
	WS	14 ± 1.7a	21 ± 2.1b	21±2.0b
PTN	WW	12 ± 2.1a	9 ± 0.6a	11±3.5a
	WS	11 ± 1.2a	15±3.5ab	16±1.0b
TG	WW	845 ± 120.4a	556 ± 38.6b	564 ± 125.8b
	WS	511 ± 86.3a	628 ± 183.1a	606 ± 143.5a
FG	WW	568 ± 177.7a	434 ±49.0a	284 ± 135.6a
	WS	321 ± 29.9a	231 ± 147.3ab	100 ± 73.0b
%EG	WW	32.78 ± 20.044ab	21.94 ± 3.556a	49.64 ± 14.039b
	WS	37.18 ± 16.668a	63.22 ± 28.495b	83.49 ± 9.399b
Biomass (g)	WW	103.52 ± 14.149a	64.98 ± 4.806b	65.34 ± 6.979b
	WS	77.23 ± 9.017a	74.88 ± 15.813a	66.95 ± 4.464a

Note: PH: plant height, TN: Tiller number, PTN: Productive tiller number, TG; total grains, FG: filled grains, EG: empty grains, SW1000: weight of 1000 seeds. * means sharing similar letter in a line do not differ significantly at $\alpha = 0.05$

Phylogenetically, *OsHox*-6 transcription factor is closely related with rice *OsHox*-22 and *OsHox*-24, and with *Arabidopsis AtHb*-7 and *AtHb*-12 as they are grouped in a same clade (Agalou *et al*. 2008, Zhang *et al.* 2014, Capella *et al*. 2016, Ribone *et al.* 2015). Presently, the *OsHox*-6 transcription factor has been known to share homology with the other genes in tomato (*Solanum lycopersicum*) *SlHZ*-20, -32, -41 and -05 (Zhang *et al.* 2014); sunflower (*Helianthus annuus*) *Hahb*-11 and *Hahb*-4 (Ribone *et al.* 2015); wheat (*Triticum*

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aestivum) *TaHDZ*-8 dan -20 (Yue *et al.* 2018); and cassava (*Manihot esculenta*) *MeHDZ*-37, -38, -39, and -41 (Ding *et al*. 2017). These genes have been identified to be responsive to some abiotic stresses including drought. However, specific functions of most of these HD-Zip genes are still unknown.

Functional analyses of these homologous genes showed that they shared similar functions but also have specificities. Functional analysis studies showed that HD-Zip transcription factors act as negative growth regulators in response to drought. Rice *OsHox*-22 and its paralog, *OsHox*-24 for example, has a function as a negative growth regulator, specifically in root elongation in response to water stress via ABA-mediated signaling pathway. Overexpression of *OsHox*-22 increased sensitivity to drought. Down regulation of *OsHox*-22, on the other hand, decreased sensitivity to ABA and increased drought tolerance. (Zhang *et al*. 2012, Bhattacharjee *et al.* 2016). *Arabidopsis AtHB*-7 and its paralog *AtHB*-12 also play a role as negative growth regulators in response to drought via ABA-mediated signaling pathway. Overexpression of both *AtHB*-7 and *AtHB*-12 reduced stem and leaf elongation, delayed senescence processes, and reduced stomatal conductance in mature plants (Olsson *et al*. 2004, Hjellstrom *et al*. 2003). Sunflower *Hahb*-4 plays a role as a negative regulator in cell elongation. Overexpression of *Hahb*-4 caused the plant had shorter stems and internodes, but became more tolerant to drought without yield penalty (Dezar *et al*. 2005). Overexpression *Hahb*-11 also induced root elongation, stem expansion and vascular bundle formation, increased plant tolerance to water stress. Since the overexpression of *OsHox*-6 did not significantly reduce plant height, this indicated that *OsHox*-6 might not play a role in stem elongation. Previous studies on the fusion of a GUS promoter with rice *OsHox*-6 and *Arabidopsis AtHB*-7, respectively, showed that GUS activities were strongly induced by water stress (Hjellstrom *et al*. 2003, Rahmawati 2012). GUS activities of *Arabidopsis* AtHB7::GUS plants were most intense at the young part of inflorescences and the elongating parts of the stems (Hjellstrom *et al*. 2003). Whereas in rice *OsHox*-6::GUS plants, GUS activities were most intense in the divisional zone of stem nodes (Rahmawati, 2012).

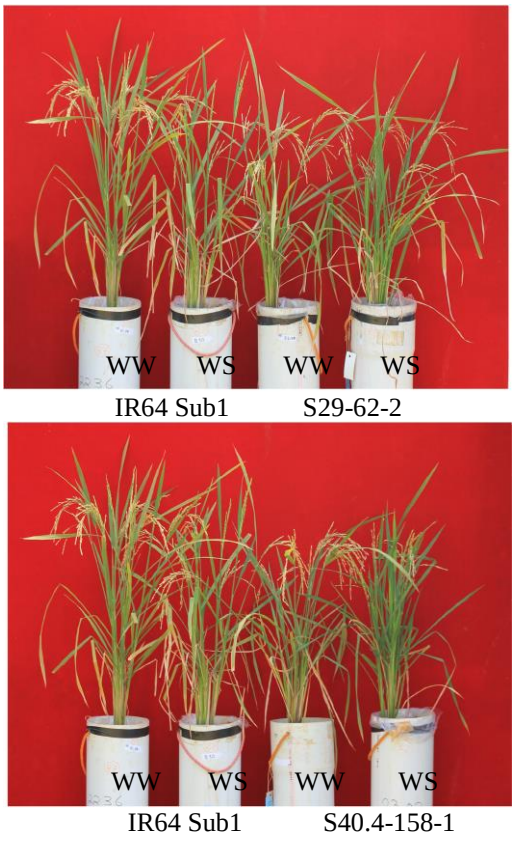

Figure 4. Comparison of transgenic rice lines overexpressing *OsHox*-6 and IR64 Sub1 control under well-watered (WW) and water stress (WS) conditions.

Overexpression of *OsHox*-6 did not significantly increase tolerance to water stress.

Based on our data, there was no significant difference between the transgenic overexpressing *OsHox*-6 and its control wild type in the water loss rates at the vegetative stage. No significant differences were also observed in the leaf water contents and drought resistance indeces (DRI) at the generative stage (Table 3). These results indicated that the overexpression of *OsHox*-6 might not significantly increase rice tolerance to the water stress. Furthermore, the overexpression of *OsHox*-6 delayed leaf senescence and caused yield penalty. These results were similar to what were observed in the other *OsHox*-6 homologs such as rice *OsHox*-22 and its ortholog *OsHox*-24, and also *Arabidopsis AtHB*-7 and its ortholog *AtHB* -12. The overexpression of *OsHox*-22, *OsHox*-24, *AtHB*-7 and *AtHB*-12 increased plant sensitivity to ABA and water stress, however, the decrease in their transcripts reduced

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sensitivity to ABA and increased tolerance to drought (Hjellstrom *et al*. 2003, Olsson *et al*. 2004, Zhang *et al.* 2012, Bhattacharjee *et al*. 2016). Thus, a down regulation or a knockdown of *OsHox*-6 expression is required to get a more comprehensive understanding on the role of the *OsHox*-6 gene in rice and its detailed mechanisms in response to drought and other abiotic stresses. Regulation of the *OsHox*-6 gene expression might be potential as a method to improve drought tolerance in rice and to shape rice architecture.

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