Research Article

Agrobacterium Mediated Transformation of *OsTZF8* Gene in *Oryza sativa* for Drought Stress Tolerance

Muhammad Medrar Hussain*, Asad Jan* and Sayyar Khan Kazi

Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Postal Code 25130, Pakistan.

Abstract | Rice (*Oryza sativa* L.) is a commonly consumed crop nourishing more than half of the human population. Different environmental stresses greatly affect cash crops. Among these, drought is a serious abiotic stress effecting rice productivity. Therefore, the current study was designed to know the role of *OsTZF8* gene in conferring tolerance to drought stress. For this purpose, *OsTZF8* gene was over-expressed in *Oryza sativa* subspecies indica variety Swat-1. Through *Agrobacterium* mediated transformation, *OsTZF8* gene (pBIG-*Ubi*-*OsTZF8*) was successfully transferred to rice calli. From these rice calli transgenic plants were regenerated and established in greenhouse. For the evaluation of *OsTZF8* gene role in drought stress tolerance, two weeks old rice seedling were subjected to drought stress. *OsTZF8*-OX transgenic indica line A and B displayed 69% and 64% survival rates respectively compared to 33% of control. These results confirmed that due to overexpression of *OsTZF8* gene, transgenic line A and B displayed considerable level of drought stress tolerance. Additionally, the chlorophyll content of top four leaves of *OsTZF8*-OX transgenic indica line A and B were observed to be high compared to control. In conclusion our study revealed that over-expression of *OsTZF8* gene has significant role in conferring tolerance to drought stress in rice.

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***Correspondence** | Muhammad Medrar Hussain and Asad Jan, Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, Khyber Pakhtunkhwa, Pakistan, Postal code 25130; **Email:** medrarbiotech@gmail.com; janasad@aup.edu.pk
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Introduction

Different biotic and abiotic stresses seriously affect plants which ends in reduced productivity (Kim et al., 2014). Drought is a serious abiotic problem related to decline crop production in different regions of the world. Non-availability of water hampers plant development and growth due to low photosynthesis rate and decline of essential nutrients (Chinnusamy et al., 2004). Worldwide, area affected by drought is gradually increasing and more than 20% of rice growing area is suffering from drought stress (Swamy and Kumar, 2013). At seedling stage, drought stress can slow down plant establishment and decrease seedling survival rate. At vegetative stage, drought can cause decrease in yield by lowering the leaf formation rate and panicle per plant, while at reproductive stage, drought results in increased grain sterility and decreased grain per panicle (Pantuwan et al., 2002).

Drought stress alters the expression of various genes that play a significant part in stress response and tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; Todaka et al., 2015). There are two main groups of stress inducible genes. The 1st group consists of those genes which encode proteins required for cellular stress

tolerance such as molecular chaperons, antifreeze proteins, soluble sugars, water channel proteins and detoxifying enzymes. The 2nd group comprises of those genes which encode proteins which are linked to signal transduction and expression of abiotic stresses related genes (Shinozaki and Shinozaki, 2007) which includes calmodulin binding proteins, components of ABA (abscisic acid) signaling, enzymes involved in metabolism, kinase proteins and transcriptional factors (Yamaguchi-Shinozaki and Shinozaki, 2006).

The plant hormone ABA has a key role in plant adaptation during drought stress. Under drought stress, ABA accumulates due to induction of ABA biosynthetic genes (Iuchi et al., 2001). The expression of many genes are regulated by ABA that leads to important changes which assist plant survival during drought stress (Umezawa et al., 2010). Under drought stress, genomic and molecular investigation have shown the presence of both ABA-dependent and ABA-independent signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki, 2006). During drought stress, several transcription factors function in ABA-dependent regulation of gene expression while some function in ABA-independent regulation of gene expression (Nakashima et al., 2009).

Upon exposure of plants to abiotic stresses, a range of different genes with various functions like production of tolerance proteins etc., are either up regulated or down regulated (Lata and Prasad, 2011). Transcriptional factors are essential regulatory proteins which direct the expression of several defense genes (Chen and Zhu, 2004). These transcriptional factors act on conserved *cis* acting sequences in the promoter region of many stress linked genes and increase the expression of a complex gene network connected with conferring tolerance against various stresses. In genetic engineering, these transcriptional factors are powerful tools for the overexpression of complete set of genes. A number of transcriptional factors are associated with the expression and regulation of many stress responsive genes. For instance, about 1500 different genes code for different transcriptional factors in *Arabidopsis* (Riechmann et al., 2000). Several large groups of transcriptional factors are classified in to different families like bZIP, AP2/ERF, zinc finger, MYB, NAC and MYC (Umezawa et al., 2006).

It has been reported that transcription factors are involved in different physiological activities under drought and other abiotic stresses. For instance, ZAT18 (C_2H_2 zinc finger protein) was transcriptionally induced by drought stress in *Arabidopsis*. In comparison to wild type, *ZAT18* overexpression plants displayed lower content of ROS (Reactive Oxygen Species), less leaf water loss, higher antioxidant enzyme activities and higher leaf water content after drought treatment (Yin et al., 2017). Another C_2H_2 zinc finger protein called IbZFP1 was reported in drought-tolerant sweet potato (*Ipomoea batatas* (L.) Lam.) line Xu55-2. Analysis showed that significant increase of soluble sugar, total chlorophyll content, ABA, proline, and reduction of malonaldehyde content and H_2O_2 were detected under drought and salt stresses in the transgenic plants. Furthermore, the rates of water loss and electrolyte leakage in transgenic plants were reduced (Wang et al., 2016). Similarly, ZAT6 (C_2H_2-type zinc finger protein) was highly enhanced by drought and salt stress in leaves of in *Arabidopsis*. Seed germination was greatly improved in overexpressed ZAT6 transgenic plants under stress conditions (Liu et al., 2013)

A diverse transcriptional factor family known as zinc finger proteins, are reported to be involved in several mechanisms like regulation of transcription, RNA binding and protein-protein interactions related to the growth and development of plants (Yilmaz and Mittler, 2008). In these cellular mechanisms of plants, different zinc finger families like ERF, LIM, WRKY, RING-finger and DOF are involved (Lijavetzky et al., 2003).

One of these important zinc finger proteins families is C_3H zinc finger protein family. In rice, full genome investigation of C_3H zinc finger proteins has revealed 67 genes (Wang et al., 2008). It has been identified that some members of these families are involved in stress tolerance against abiotic stresses. For example, a C_3H zinc finger protein gene *OsTZF1* (*Ubi*:*OsTZF1*-OX), exhibited better tolerance to drought and high NaCl conditions (Jan et al., 2013). Likewise, OsC_3H_{47} (C_3H zinc finger protein gene) was reported to induce strongly under ABA, NaCl, PEG and osmotic stresses. The over expression of this gene in rice seedlings exhibited enhanced tolerance to salt and drought stresses (Wang et al., 2015).

A small subfamily of zinc finger proteins called Tandem C_3H Zinc Finger (TZF) proteins is characterized by the presence of Tandem Zinc Finger domains which

are separated by 17 amino acids. These 17 amino acids are actually involved in RNA binding process. TZF proteins had been reported as RNA-binding proteins having significant role in RNA processing (Jeong et al., 2010). Characterization of these genes specified their role in giving tolerance against various abiotic stresses like cold, drought, salt etc. Some C_3H zinc finger proteins members have been identified to have essential roles in plant growth and architecture (Wang et al., 2008; Zhang et al., 2015; Liu et al., 2017; Yin et al., 2017).

Through genetic engineering, rice varieties tolerant to abiotic stresses can be produced by incorporating genes involved in abiotic stresses. For improved tolerance to adverse environmental conditions, such genes could be incorporated into plants through different gene transfer methods like particle bombardment, electroporation and *Agrobacterium*-mediated transformation. The most extensively practiced gene transfer method in rice is *Agrobacterium*-mediated transformation (Gelvin, 2003). In *Agrobacterium* based transformation, one or few copies of desired gene is transferred with high efficiency into plant genome (Wu et al., 2003). Fruitful attempts have been made to infect monocotyledons with *Agrobacterium*. Successful transformation of cereal crops via *Agrobacterium* has been successfully carried out in maize, wheat and rice (Shen et al., 1999).

Most of the members of TZF gene family in rice have not been functionally characterized. One uncharacterized member of C_3H zinc finger protein genes is OsC_3H_{10} which is also known as *OsTZF8* having a unique TZF motif C-x_{10}-C-x_5-C-x_3-H along with another common motif C-$_{X5}$-C-$_{X4}$-C-$_{X3}$-H. In this research study, we have evaluated the role of *OsTZF8* gene in conferring drought stress tolerance. *OsTZF8* gene was over-expressed in rice indica variety Swat-1. *Agrobacterium* mediated transformation was used for *OsTZF8* gene transfer into rice calli. Chlorophyll content and morphological parameters of transgenic rice were also evaluated.

Materials and Methods

Plant material and growth conditions

Seeds of *Oryza sativa* subspecies indica variety Swat-1 was used in the present study. Seeds were acquired from the Plant Genetic Resource Institute (PGRI), National Agriculture Research Council (NARC), Islamabad. For experimental purposes rice seeds were incubated for 3 days at 42°C in oven (Hanumanthappa et al., 2016).

Transformation of pBIG binary vector having Ubi-OsTZF8 construct into Agrobacterium

Agrobacterium mediated transformation was used for transferring *OsTZF8* gene (pBIG-*Ubi-OsTZF8*) into rice for overexpression (Rashid et al., 1996; Toki, 1997). *Agrobacterium tumefaciens* strain EHA-105 was used for rice transformation. Binary plant expression vector pBIG (12.7kb) was used as backbone for construction of binary vector containing *OsTZF8* coding region under the maize ubiquitin (*Ubi*) promoter and hygromycin phosphotransferase (*HPT*) marker gene. Freeze/thaw method was used for the transformation of *Agrobacterium tumefaciens* (EHA-105) with pBIG-*Ubi-OsTZF8* construct (Wise et al., 2006). Plasmid DNA (1 µg) was added to 1.5 ml eppendorf tube containing *Agrobacterium* competent cells and mixed by tapping. Tubes were frozen in liquid nitrogen for 2 min and then thawed at 37°C for 5 min. Thawed bacterial cells were then mixed with 1 ml LB media and incubated in shaking incubator at 28 °C for 2 hrs. Centrifugation was done at 4000 rpm for 5 min and supernatant was discarded. Pellet was re-suspend in 100 µl LB. The bacterial suspension was spread on LB media plats having kanamycin (100 mg/L), hygromycin (50 mg/L) and rifampicin (10 mg/L) and incubated at 28°C for 2 days.

Confirmation of transformed Agrobacterium colonies

After 2 days of selection on antibiotics supplemented LB media, Colony PCR was performed for confirmation of pBIG-*Ubi-OsTZF8* construct in colonies of *Agrobacterium* (Sheu et al., 2000). PCR was done using construct specific forward (*Ubi*-F1) and reverse (*OsTZF8*-R200) primers.

Rice calli infection with transformed Agrobacterium tumefaciens

For the transformation of rice calli with pBIG-*Ubi-OsTZF8* construct, mature and healthy seeds of Swat-1 indica rice were dehusked and washed with tape water, 0.2% $MgCl_2$ solution (3 times) and sterile distilled water (5 times) for 1 minute each. For the purpose of calli induction, fourteen seeds were cultured on callus induction media (N6) (Chu et al., 1975) for 21-28 days at 25±3 °C. The procedure of Rashid et al. (1996) and Toki et al. (1997) was followed for rice transformation. Previously transformed *Agrobacterium tumefaciens*

strain EHA-105 was used for the transformation of induced rice calli. *Agrobacterium* culture in a 50 ml falcon tube containing 30 ml AA-medium (Toriyama and Hinata, 1985) supplemented with acetosyringone (10 mg/L) was used. Before infection, viable rice calli were collected in a sterilized sieve and infected with the above prepared *Agrobacterium* suspension. After 90 seconds, the calli were dried and placed on co-cultivation media plates and were incubated for 3 days at 28 °C in dark. Co-cultivation medium was prepared by using N6 medium (Chu et al., 1975) constituents and supplementing it with acetosyringone (10 mg/L). After 3 days of co-cultivation, calli were washed with sucrose solution (3%) having 250 mg/ml carbencillin and then with sterile distilled water. Calli were then shifted to selection media plates and incubated at 28°C for 10 days and sub-cultured after every 10 days. For selection medium, N6 medium was prepared having kinetin and 2,4-D as growth regulators. After addition of 1% agar, the media was autoclaved. The medium was then supplemented with 250 mg/L carbencillin and 30 mg/L hygromycine. After selection, calli were shifted to regeneration medium for regeneration. The ingredients of regeneration medium were MS salts with vitamins (Murashige and Skoog, 1962) 4.43g/L, sucrose and D-sorbitol 30g/L each, casamino acid 1g/L, NAA (naphthalene acetic acid) 0.02 mg/L, kinetin 4 mg/L, hygromycine 30 mg/L, carbencillin 500 mg/L and agar 3 g/l (solidifying agent). In regeneration medium calli were permitted to regenerate in light at 28°C (16 hrs light and 8 hrs dark photoperiod). Certain embryogenic rice calli turned green from which small plantlets regenerated.

PCR confirmation of transformed rice calli

For DNA extraction from putative transformed rice calli, Phillips et al. (2001) protocol was followed. Genomic DNA was isolated by taking 0.1 g of calli in tube and crushing in liquid nitrogen and transferred to eppendorf tubes. To each tube, 400 µl EB_2 (extraction buffer) solution was added and mixed vigorously for 5 min. Then 200 µl (20%) sodium dodecyl sulphate (SDS) was added to each sample and was mixed for 3 minutes. About 1 µl RNase was added to each tube and was incubated at 37°C for 30 min. To each sample, 400 µl phenol mix was added and centrifuged at 14000 rpm for 5 min. To a fresh tube, supernatant was transferred and 400ul isopropanol was added and centrifuged at 10,000 rpm for 1 min. The pellet containing DNA was washed with 70% ethanol and then dried. The pellet was suspended in 10-30 µl TE (Tris EDTA) buffer for further use. Concentration and quality of isolated DNA was verified through Nano drop (2000/2000C, Thermo scientific, USA). For the confirmation of amplified region in calli DNA, PCR investigation was performed. The PCR product was examined using 1% agarose gel with 1kb plus Ladder and visualized in UV trans-illuminator.

Establishment and acclimatization of transgenic indica rice

Small plantlets were regenerated from selected green calli in regeneration media plates and jars. Then these rice plantlets were shifted for establishment to full and half MS media (Murashige and Skoog, 1962) supplemented with hygromycin (30 mg/L). Plantlets were first shifted to small pots having sterile soil and kept under standard growth conditions at 28°C and 55% humidity and then to green house for acclimatization and maturity.

PCR confirmation of transformed indica rice plants

Molecular level confirmation of the transgenic rice plants having *OsTZF8*-OX was carried out using PCR. DNA was extracted from the young transgenic rice leaves using Phillips DNA extraction protocol (Phillips et al., 2001). PCR confirmation of *OsTZF8* and *HPT* genes were performed using the DNA extracted from transgenic rice lines.

Drought stress treatment of indica OsTZF8-OX transgenic rice

Rice plant seedlings of *OsTZF8*-OX transgenic indica rice were grown for two weeks in soil and then subjected to dehydration stress. After the dehydration stress treatment, plants were permitted to recover in normal growth conditions (28°C and 55% humidity). The number of plants survived and continued to grow after two weeks were counted (Jan et al., 2013).

Chlorophyll and Morphological investigation of indica OsTZF8-OX transgenic rice

Chlorophyll content of *OsTZF8*-OX transgenic rice leaves was measured through Spad meter (Spectrum technologies, USA). To examine the effects of overexpression of *OsTZF8* gene, chlorophyll content of *OsTZF8*-OX transgenic and control rice plants was compared. For this purpose, chlorophyll content was measured from upper, middle and lower portion of first four leaves of rice plants (Wang et al., 2016).

Morphological data like height of the plant, number

of tillers, number of total grains, number of filled grains and % yield was collected from *OsTZF8*-OX transgenic rice plants (Ogunbayo et al., 2005).

Statistical analysis

For the experiments of drought stresses, chlorophyll content etc, the mean values were calculated and diagrammatically represented as bar charts using Microsoft Excel program. To know the treatment efficiency, t-test was applied to various experiments as described by Steel and Torrie (1960); $P < 0.05$ was considered the minimum value for statistical significance.

Results and Discussion

pBIG-Ubi-OsTZF8 construct map

For the *OsTZF8* gene overexpression in rice, a suitable construct was prepared using pBIG binary vector. The construct have *OsTZF8* gene under ubiquitin promoter (*Ubi*) and hygromycin phosphotransferase (*HPT*) gene under *Nos* promoter (Figure 1).

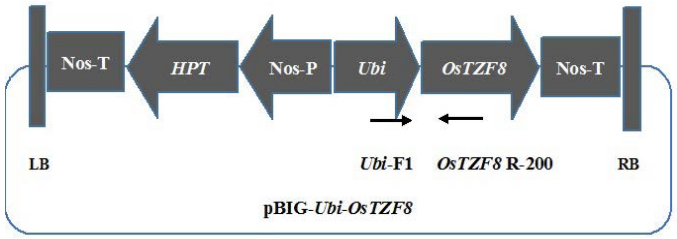

Figure 1: *pBIG-Ubi-OsTZF8 construct map having OsTZF8 gene and selectable marker gene hygromycin phosphotransferase (HPT). Total size of pBIG-Ubi-OsTZF8 construct was 14249 bp.*

Agrobacterium tumefaciens transformation and colony PCR confirmation

Competent cells of *Agrobacterium tumefaciens* were transformed with pBIG-*Ubi-OsTZF8* construct. Few transformed *Agrobacterium* colonies were obtained on LB selection media plates having kanamycin, hygromycin and rifampicin antibiotics. For the confirmation of transformed colonies, Colony PCR was performed. For this purpose, five colonies of *Agrobacterium tumefaciens* were selected and there DNA was subjected to PCR. PCR was performed using construct specific forward (*Ubi*-F1) and reverse (*OsTZF8*-R200) primers (Table 1). After Colony PCR, three colonies exhibited positive bands on 1% agarose gel, confirming the primers specific amplification of the *OsTZF8* gene with band size of approximately 300 bp (Figure 2). Colony PCR confirmed *Agrobacterium* colonies were selected and successfully revived.

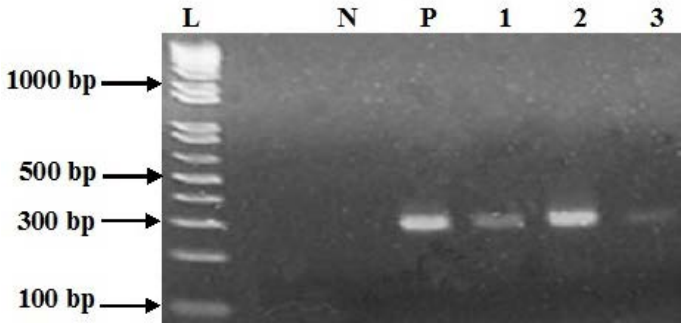

Figure 2: *PCR confirmation of Agrobacterium (EHA-105) colonies transformed with pBIG-Ubi-OsTZF8; Lane 1-3: DNA from Agrobacterium colonies; P) Positive control; N) Negative control; L) Ladder.*

Agrobacterium mediated transformation of rice calli with OsTZF8 gene and its PCR confirmation

About 14 seeds of indica rice were used per callus induction media plate (total seeds used were 397). A total of 280/360 calli were found suitable for transformation. After successful transformation, 24 calli were selected having optimum regeneration characteristics. After several plantlets germination, out of 9 germinated transgenic plants 2 lines were selected for the current study (Table 2). Genomic DNA from indica rice calli was isolated and analyzed through PCR using construct specific forward (*Ubi*-F1) and reverse (*OsTZF8*-R200) primers (Table 1). Results of amplified samples on PCR along with positive control were observed on 1% agarose gel. Seven samples (1 to 7) showed amplification of *OsTZF8* gene inserted into rice calli. The band size of samples and positive control was approximately 300 bp (Ladder size = 1kb plus) using PCR specific primers (Figure 3). The amplification of DNA bands confirmed the successful transformation of *OsTZF8* gene into rice calli.

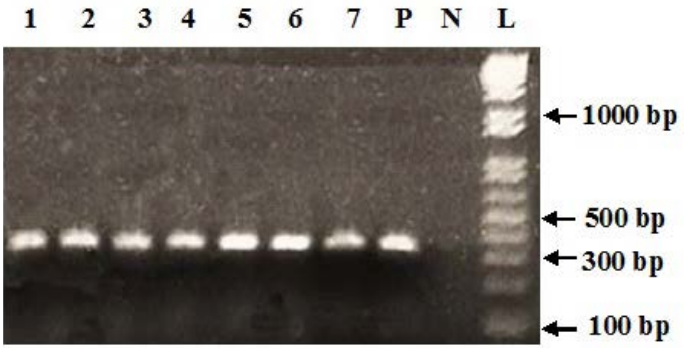

Figure 3: *Gel electrophoresis of PCR amplified DNA bands of transformed calli having pBIG-Ubi-OsTZF8 construct. Lane 1 to 7: DNA samples from different calli; P) Positive control; N) Negative control; L) Ladder.*

Table 1: *List of primers used for confirmation of OsTZF8 and HPT genes.*

Gene name	Primer type	Primer name	Primer sequence
OsTZF8	Forward	*Ubi*-F1	5´-TGCTCTAACCTTGAGTACCTATTAT-3´
	Reverse	*OsTZF8*-R200	5´-GGGCACGACGTCCACTCGTGCGCCGCGA- 3´
Hygromycin phospho-transferase (*HPT*)	Forward	HPT-F	5´-ATGAAAAAGCCTGAACTCACCGCGA-3´
	Reverse	HPT-R	5´-GCAAAGTGCCGATAAACATAACGATC-3´

Plants regeneration and acclimatization

After PCR confirmation of *OsTZF8* in rice calli, transformed rice plants were regenerated from these calli on full MS and half MS media having hygromycin antibiotic as selection marker. The plants were first grown on media plates, then transferred to media jars and then gradually transferred to small and large pots having sterile soil. Finally, the mature transgenic plants were grown successfully in green house under control conditions (Figure 4).

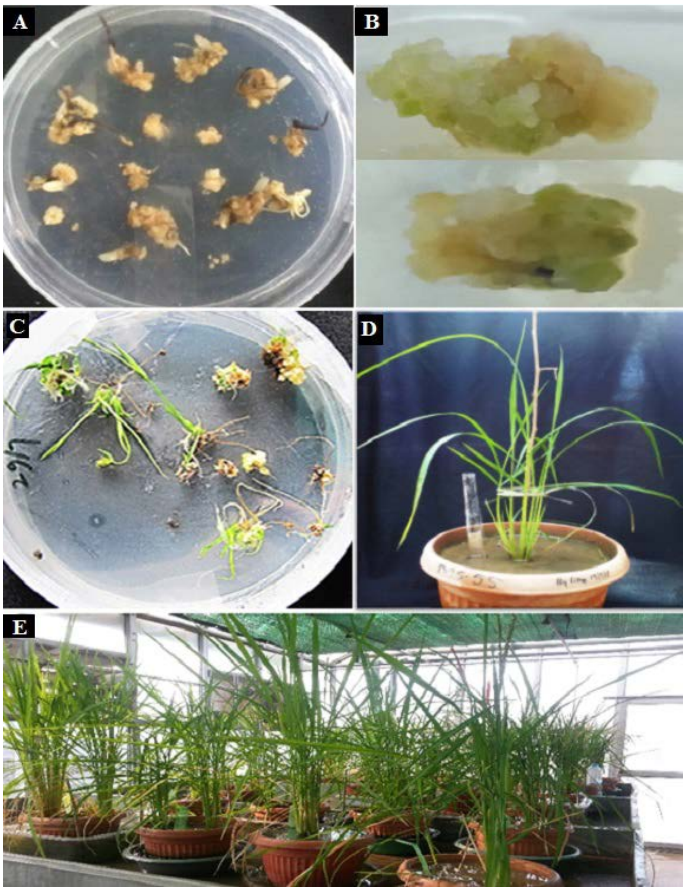

Figure 4: *Different stages of OsTZF8-OX transgenic indica rice plants generation; (A) Callus induced from rice seeds; (B) Calli regeneration; (C) Plantlets regeneration; (D) Plants transferred to soil; (E) Plants transferred to green house for acclimatization.*

Molecular level confirmation of OsTZF8-OX and HPT gene in transgenic rice lines

Genomic DNA was isolated from indica *OsTZF8*-OX transformed plantlets. By using *Ubi*-F1 and *OsTZF8*-R200 primers (Table 1), PCR confirmation was performed. Transformed rice plants DNA showed positive bands at 300 bp along with positive control (Figure 5A). The amplification of DNA bands indicated the successful transformation of *OsTZF8* gene into indica rice plants.

Table 2: *Summary of transformation and regeneration of rice calli.*

Seeds per plate (callus induction media)	14
Total seeds used for calli formation	397
Total number of calli regenerated	360
Number of calli used for transformation	280
Number of transformed calli having regeneration capacity	24
Number of germinated transgenic plants	9
Rice transgenic lines selected for research work	2

After confirmation of *OsTZF8* gene in rice, the inserted transcript was also confirmed for the presence of *HPT* on PCR using specific primer (Table 1). Positive bands were detected around 200 bp along with positive control (Figure 5B). This result gives an additional confirmation of successful transformation of the transgenic pBI vector into rice.

Drought stress analysis of indica OsTZF8-OX transgenic lines

For the evaluation of drought stress tolerance, two weeks old rice seedlings of *OsTZF8*-OX transgenic lines were exposed to drought stress. Plants were recovered under normal growth conditions after stress treatment. *OsTZF8*-OX transgenic line A and B exhibited 69% (25/36) and 64% (23/36) survival rates respectively in comparison to 33% (12/36) of control (Figure 6). These findings confirmed that *OsTZF8*-OX line A and B revealed considerable level of tolerance to drought stress compared to control rice plants.

Chlorophyll content study of OsTZF8-OX transgenic rice lines

Chlorophyll content measurement was carried out at 4 and 8 weeks after heading (WAH). After 4 WAH,

Table 3: *Morphological investigation of mature indica OsTZF8-OX transgenic rice line A, B and control. Results are the mean ± SD of at least three replicates.*

Rice lines	Plant height (cm)	± SD	Tillers per plant	± SD	Grains per panicle	± SD	Filled grains per panicle	± SD	%Yield	± SD
OsTZF8-OX A	84	1.37	18	1.79	110	5.39	97	2.73	88.18	1.92
OsTZF8-OX B	86	0.89	16	0.52	96	5.09	88	3.61	91.66	2.98
Control	95	2.73	15	2.68	94	4.50	79	3.58	84.04	2.23

the chlorophyll content of four leaves (top to bottom) of *OsTZF8*-OX A and B transgenic lines were observed to be high compared to control. Similarly, After 8 WAH, chlorophyll content of *OsTZF8*-OX transgenic line A and B was high in comparison to control (Figure 7).

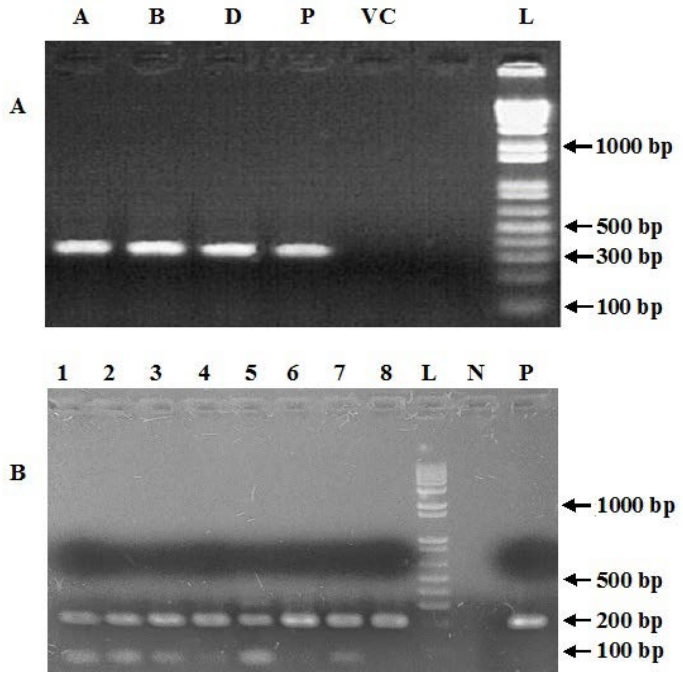

Figure 5: *Molecular confirmation of OsTZF8-OX and HPT genes in indica rice; (A) Gel electrophoresis of PCR amplified samples of indica OsTZF8-OX transformed plants; Lane A, B, D: DNA bands of transgenic indica rice; (B) Gel electrophoresis of PCR amplified DNA bands of transgenic rice plants having HPT gene; Lane 1 to 8: DNA samples from different calli; P) positive control; VC) Vector control; N) negative control; L) Ladder.*

Morphological investigation of mature plants

To know the effects of *OsTZF8* over-expression, morphological investigation of *OsTZF8*-OX transgenic rice plants was performed. *OsTZF8*-OX transgenic line A and B showed plant height of 84±1.37 cm and 86±0.89 cm respectively compared to 95±2.73 cm of control plant. There were 18±1.79 and 16±0.52 tillers in indica *OsTZF8*-OX transgenic rice line A and B, respectively compared to 15±2.68 of control plant. The % yield was recorded to be 88.18±1.92 and 91.66±2.98 for indica *OsTZF8*-OX transgenic rice line A and B compared to 84.04±2.23 of control respectively (Table 3).

Rice is a widely consumed staple food crop grown over more than 162 million hectares worldwide with 759.6 million tons annual production (FAO, 2018). It is a common cash crop nourishing more than half of the human population (Benjamin and Nielsen, 2006). Different abiotic stresses pose severe threats to rice crop by affecting plant growth, plant productivity and biomass production. Drought is one such environmental stress effecting rice productivity (Chinnusamy et al., 2004; Rodziewicz et al., 2014). In sever abiotic stresses, plants usually survive and grow by developing several molecular, physiological, biochemical and morphological changes. Upon exposure of plants to these stresses, various genes with different functions are either up regulated or down regulated (Shinozaki and Shinozaki, 2007; Lata and Prasad, 2011). Transcriptional factors are essential regulatory proteins having key roles in the expression of several defense genes (Chen and Zhu, 2004). The zinc finger protein genes is one of the major transcription factors family which is mainly involved in various mechanisms of plant growth and development such as protein-protein interactions, RNA binding and Transcription regulation (Yilmaz and Mittler, 2008). CCCH zinc finger protein family is an important tandem zinc finger (TZF) proteins family having key roles in abiotic stress tolerance. In rice, full genome study of CCCH zinc finger proteins have displayed 67 genes (Wang et al., 2008). One uncharacterized member of CCCH zinc finger protein genes family is *OsTZF8* which is also called OsC_3H_{10}. In the present research work *OsTZF8* genes was transferred to indica rice through *Agrobacterium tumefaciens* to find out its role in drought stress tolerance.

To know the role of *OsTZF8* gene in drought stress tolerance, a suitable construct pBIG-*Ubi-OsTZF8* was prepared (Figure 1). pBIG-*Ubi-OsTZF8* construct

was used for the overexpression of *OsTZF8* in rice. First of all the competent cells of *Agrobacterium* were prepared and transformed with the pBIG-*Ubi-OsTZF8* construct. Then the transformed colonies were confirmed through colony PCR (Figure 2). Successful *Agrobacterium* mediated transformation of rice calli with *OsTZF8* was carried out through co-cultivation, selection and regeneration steps using different media supplemented with antibiotics such as carbencillin and hygromycin. The transformed rice calli were confirmed through PCR (Figure 3). *Agrobacterium* mediated transformation has been effectively used in producing transgenic rice lines previously (Hiei et al., 1994; Dong et al., 1996; Jan et al., 2013). Rashid et al., (1996) successfully applied this method to produce transgenic indica rice (Basmati cultivars). Similarly, Aldemita and Hodges (1996) reported transformation in young embryos of japonica and indica varieties using of *Agrobacterium tumefaciens*. Furthermore, rice calli has been used successfully to transfer desired genes through *Agrobacterium*. For instance, Jan et al. (2013) used japonica rice calli to transfer *OsTZF1*gene using *Agrobacterium*-mediated transformation. Hiei et al. (1994) also used *Agrobacterium*-mediated transformation in japonica rice calli to produce transgenic plants which was confirmed at molecular level. PCR level confirmation is a reliable technique and used in routine for the confirmation of genes transformation. For example, PCR confirmation of transgenic calli was carried out for manganese superoxide dismutase (*Mn-SOD*) gene in rice responsible for salt stress tolerance (Wang et al., 2007). Similarly engineered cysteine proteinase inhibitor (Oryza cystatin-IDD86) for nematode resistance in transgenic rice plants were confirmed through PCR (Vain et al., 1998). After successful *Agrobacterium* mediated transformation of *OsTZF8* gene into rice calli, we regenerated and established transformed rice plants in green house (Figure 4).

Molecular level verification is a key step in confirmation of successful transfer of desired gene into the host cells (Sarker et al., 2015; Cui et al., 2017; Pradhan et al., 2017). In the present study genomic DNA from transformed indica rice plants was extracted through Phillips et al. (2001) method and confirmation of pBIG-*Ubi-OsTZF8* construct along with *HPT* gene was performed through PCR (Figure 5A and B). The positive DNA bands amplification indicated the successful transformation of *OsTZF8* gene into rice plants. Genomic DNA of transgenic plants has also been used previously in PCR to confirm successful integration of transgene. For instance, gene insertion of *AtDREB1A* gene (responsible for gene regulation in drought stress) in rice plants genome was also confirmed through PCR (Hussain et al., 2014). Similarly, *OsCYP2* gene responsible for root development in rice was also confirmed from genomic DNA through PCR (Cui et al., 2017). Constitutive expression of *OsDof4* gene which codes a C_2-C_2 zinc finger transcription factor was also confirmed initially through PCR using genomic DNA (Wu et al., 2017).

Drought is an abiotic stress which severely damage crops production in different regions of the world (Chinnusamy et al., 2004). CCCH zinc finger proteins is a transcriptional factor family which conferred tolerance to many abiotic stresses like drought (Zhang et al., 2013; Pradhan et al., 2017). Therefore, we evaluated *OsTZF8* gene role in drought stress tolerance in rice. For this purpose, two weeks old rice seedlings were subjected to dehydration stress. *OsTZF8*-OX transgenic line A and B exhibited 69% and 64% survival rates, respectively compared to 33% in control (Figure 6). Our results suggests the positive role of *OsTZF8* gene in conferring drought stress tolerance. In line to our results, previous findings also suggests key roles of stress inducible genes in drought stress tolerance. For example, overexpression of a CCCH gene called *OsTZF1*in japonica rice showed improved tolerance to drought stress. Japonica *OsTZF1*-OX line #6 and #9 exhibited 69% and 63% survival rates respectively compared to 38% in control (Jan et al., 2013). Likewise, the overexpression lines #2 and #3 of *ONAC045* gene in rice showed 90% and 70% survival rate respectively after recovery in comparison to 35% in control (Zheng et al., 2009). In another study *OsDREB1F* gene over-expression showed high drought stress tolerance in *Arabidopsis* and rice transgenic lines compared to controls (Wang et al., 2008).

Measuring chlorophyll content also gives an indication of gene role in phenomenons such as delayed leaf senescence, drought stress tolerance etc. (Wang et al., 2005). To evaluate the effects of *OsTZF8* gene on chlorophyll, chlorophyll content of transgenic lines *OsTZF8-OX* A, *OsTZF8-OX* B and control were measured for top four leaves after 4 and 8 WAH. It was observed that all four leaves of transgenic lines retain a high chlorophyll content compared to control plants (Figure 7). These results indicated the positive role of *OsTZF8* gene in chlorophyll content of *OsTZF8*-OX

transgenic leaves. Previous studies showed that certain genes have roles in increasing or stabilizing chlorophyll content. For example, the *AtTZF1* transgenic *Arabidopsis* lines showed high levels of chlorophyll content compared to control lines (Han et al., 2014). In another study the *TaSP* gene which was cloned in *Arabidopsis* for salt stress tolerance also showed high chlorophyll content compared to control plants (Ma et al., 2015). Our findings confirms the link between over-expression of *OsTZF8* gene and tolerance to drought stress along with chlorophyll content stability/ increase, which suggests that *OsTZF8* gene might have an essential role in inducing tolerance to drought stress in rice and perhaps other crops. Overall, our findings provide evidence that *OsTZF8* gene was involved in drought stress tolerance in rice.

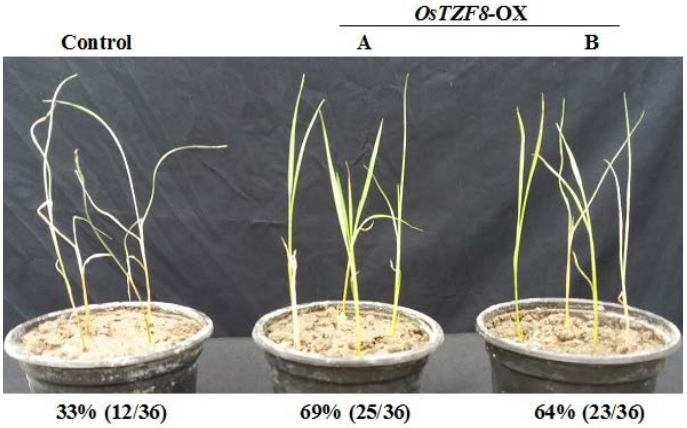

Figure 6: *Drought stress tolerance analysis in control and indica OsTZF8-OX transgenic lines. The transgenic line A (69%) and B (64%) exhibited high drought tolerance compared to control (33%) plants. t-test was used for statistical analysis (P = 0.04, P < 0.05).*

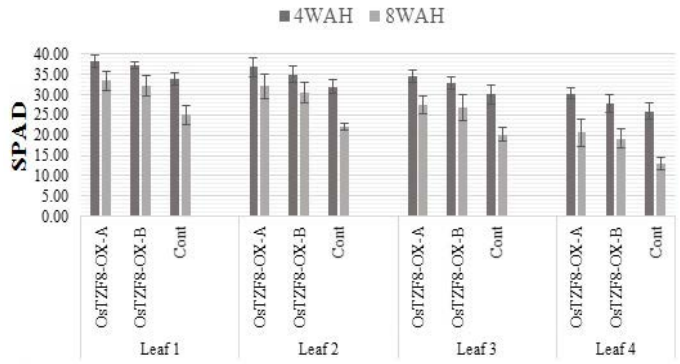

Figure 7: *Chlorophyll analysis of OsTZF8-OX transgenic indica lines and control. The measurement was carried out at 4 and 8 WAH. Due to OsTZF8 gene overexpression, all four leaves of transgenic line A and B showed high chlorophyll content in comparison to control. SPAD is the unit of chlorophyll measurement on SPAD meter.*

Conclusions and Recommendations

OsTZF8-OX transgenic indica rice lines were successfully developed through *Agrobacterium* mediated transformation. After biochemical and Molecular confirmation, the *OsTZF8*-OX transgenic lines were subjected to drought stress. Drought stress analysis indicated that *OsTZF8*-OX transgenic lines were tolerant to dehydration stress compared to control. Chlorophyll analysis also supports the current findings. Microarray and Real time PCR studies are suggested for further confirmation of *OsTZF8* gene overexpression role in drought stress tolerance. Furthermore, the transgenic lines shall be tested against other abiotic stresses.

Novelty Statement

Drought stress is a major problem for crops especially in rice. In the current study, *OsTZF8*-OX transgenic rice lines were developed for the first time in local indica rice variety of Pakistan to know its role in drought stress tolerance. Using *Agrobacterium* mediated transformation, we successfully developed *OsTZF8*-OX transgenic indica lines which exhibited tolerance against drought stress.

Author's Contribution

MMH performed the experimental work and wrote the manuscript. AJ designed and supervised the study. SKK helped in supervision and critical revision of the data and manuscript. All the authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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