

Cloning and characterization of a gene encoding novel zinc finger protein transcription factor induced under water deficit stress from rice (*Oryza sativa*) cv. N-22

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A gene *OsZn1* encoding Cys3/His1-type zinc finger protein was isolated from the water stress-induced cDNA library of rice (*Oryza sativa*) cv. N-22, an early maturing, deep-rooted, drought-tolerant genotype adapted to upland conditions. The *in-silico* analysis revealed an insert of 800 bp with an ORF of 663 nucleotides, encoding 221 amino acids. *OsZn1* had three distinct features — nuclear localization signal (NLS) present in Arg152-Arg168, Zn finger domain between 185-193 amino acids and 12 amino acids conserved domain in 71-82 amino acids homologous to LEA motif, and belonged to C-type family of Zn finger protein. *OsZn1* showed induced expression under water deficit stress.

Keywords: Drought stress, Transcription factor, Zinc finger protein, Water deficit stress, Rice, *Oryza sativa*, N-22

Plants have evolved a complex signaling network that mediates the perception of signals and responses to different environmental cues like water, salt and cold stress. A number of genes are upregulated and downregulated during water deficit stress (WDS). The knowledge of the mechanisms by which plants perceive and transduce these stress signals is the key to understanding these responses and genetic improvement of stress tolerance through transfer of key regulatory genes. Two different strategies — targeted and non-targeted can be employed to isolate differentially expressed genes with respect to WDS. Targeted approach includes amplification of known differentially expressed genes using gene-specific primers, whereas non-targeted approach involves a number of techniques such as subtractive hybridization, differential screening, cDNA-AFLP, SAGE and differential display etc.

Rice (*Oryza sativa*), one of the most important cereal crops has emerged as an ideal model species for

the study of crop genomics due to its commercial value, small genome size (430 Mb), diploid origin and close relationship with other important cereal crops. The productivity of rice is sensitive to a number of biotic and abiotic factors, wherein drought stress constitutes an important yield-limiting determinant¹. Plants respond to WDS at both cellular and molecular levels. All the genes associated directly or indirectly with stress survival and stress tolerance are mediated through abscisic acid (ABA)-dependent and independent signal pathways². These gene products can be classified into two groups. First group includes many of the water deficit-induced genes encoding gene products predicted to protect cellular functions. Second group of gene products consists of regulatory proteins that are involved in regulation of gene expression, signal transduction e.g. kinases, phosphatases and transcription factors (TFs) etc³.

Genetic engineering by employing TFs is particularly a promising strategy for stress tolerance. The potential of this strategy lies in the fact that most if not all stress-related TFs are likely to regulate a wide range of target genes that contribute to stress tolerance⁴. Therefore, the approach to engineer stress tolerance by modification of TF levels might have clear advantage over other strategies that focus on enhancing levels of individual proteins with protective function. A number of transcription elements regulating environmental stress responsive

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Abbreviations: ABA, abscisic acid; ABRE, abscisic acid responsive element; AFLP, amplified fragments length polymorphism; CRT, C-repeat; DRE, dehydration responsive element; ERF, ethylene response factor; LEA, late embryogenesis abundant; NLS, nuclear localization signal; SAGE, serial analysis of gene expression; WDS, water deficit stress; ZFP, zinc finger protein.

genes in higher plants have been isolated and characterized⁵.

Drought-inducible genes display characteristic cis-acting elements such as DRE/CRT, ABRE etc. Regulation of gene expression through DRE/CRT *cis* elements appears to be mainly ABA-independent, whereas ABRE controlled gene expression is mainly ABA-dependent. In addition to these major pathways, other regulons including the NAC, MYB/MYC, WRKY and Zn finger TF families also have important roles in response to abiotic stresses. However, recent studies have shown that cross-talk exists between ABA-dependent and independent pathways⁶. Transcriptome analysis using microarray technologies together with conventional approaches have revealed that several TFs are involved in plant response to WDS. These fall into several large TF families, such as AP2/ERF, bZIP, NAC, MYB, MYC, zinc-finger and WRKY etc.

Keeping in view the importance of TF proteins in abiotic stresses, in the present study, an attempt has been made to isolate a WDS-responsive gene encoding a regulatory protein from *Oryza sativa* L. subsp. *indica* cv. N-22, an early maturing, deep-rooted, drought-tolerant genotype adapted to upland conditions.

Materials and Methods

Plant material

Seeds of *Oryza sativa* L. subsp. *indica* cv. N-22, an early maturing, deep-rooted, drought-tolerant genotype adapted to upland conditions were procured from Genetics Division, IARI, New Delhi. They were washed with distilled water, surface-sterilized with 95% ethanol, 2% sodium hypochlorite or 0.02% mercuric chloride and germinated in the growth chamber of National Phytotron Facility, IARI, New Delhi. The seedlings were transplanted in 16 cm pots with a density of 4 plants per pot and grown under controlled conditions of 30°C/25°C (day/night) under the light intensity of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Photosynthetic photon flux density). Water stress was imposed by withholding the water supply at 40 days after germination (DAG). Relative water content (RWC) of leaf tissue was estimated as described previously⁷. Control plants were watered daily and their RWC was found to be 96-98%. When rice seedlings attained 65-85% RWC after withholding water supply, leaf tissue was harvested and used as stressed experimental material.

RNA isolation

Leaf samples were collected from control as well as stressed rice seedlings (each succeeding day starting from 85% RWC till it reached 65%) and immediately frozen in liquid nitrogen and stored till RNA isolation. Total RNA was isolated using guanidium isothiocyanate (GTC) method and subjected to electrophoresis on 1.2% agarose gel containing 20 mM GTC. mRNA was isolated from ~250 μg of total RNA from stressed leaf tissue using oligotex mRNA spin column, following the protocol supplied with Qiagen poly A⁺ RNA isolation kit (Qiagen Inc., USA).

Construction of cDNA library

cDNA was synthesized from mRNA pooled from different stages of RWC samples using SmartTM cDNA library construction kit (BD Biosciences Clontech, USA). 5 μl sample of single stranded cDNA product was checked on 1.2% ethidium bromide stained agarose gel. The cDNA (2-3 μg) was digested with *Sfi*I enzyme and size-fractionated by CROMA SPIN 400-column. cDNA in size 0.5-1.5 kb was pooled, cloned and packaged into λ TriplEx2 phage vector as per Gigapack III packaging extract kit (Promega, USA).

Conversion of λ TriplEx2 to pTriplEx2

The conversion of a λ TriplEx2 clone to a pTriplEx2 plasmid involves *in vivo* excision and circularization of a complete plasmid from the recombinant phage. The plasmid is released as a result of *cre*-recombinase mediated site-specific recombination at the *loxP* site flanking the embedded plasmid. Basically, release of the plasmid occurs automatically when the recombinant phage is transduced into a bacterial host (*E. coli* BM 25.8) in which *cre*-recombinase is being expressed. In this kit, *E. coli* BM 25.8 provides the necessary *cre*-recombinase activity. An aliquot of primary library was excised and individual single clones were selected as per manufacturer's instructions.

Plasmid DNA isolation

The plasmid DNA was isolated as per the protocol mentioned in alkaline lysis miniprep method⁸.

Probe used in study

The DNA fragment obtained by restriction digestion of plasmid DNA was labeled with [α ³²P] dATP using HexalabelTM DNA labeling kit (MBI Fermentas Inc., USA) following the manufacturer's protocol.

Northern blotting

Northern analysis was done using the RNA from control plants as well as plants grown under water deficit stress using standard protocol⁹.

Sequencing of identified clone

Automated sequencing of the putative clones was done at DNA Sequencing Facility, South Campus, Delhi University, New Delhi.

Result and Discussion

A cDNA library was constructed from mRNA isolated from stressed leaf tissue of rice *cv.* N-22 with an aim to isolate differentially expressed genes with respect to WDS. An aliquot of primary library was excised and individual single clones were selected. Six recombinant clones were selected randomly and plasmid DNA was restricted with *EcoRI* and *XhoI*, which flank the multiple cloning sit (MCS) (Fig. 1). Four of these clones revealed an insert of 800 bp. Inserts from these clones were further characterized by Northern analysis using RNA from control as well as water stressed leaf tissue. Only one of the clones

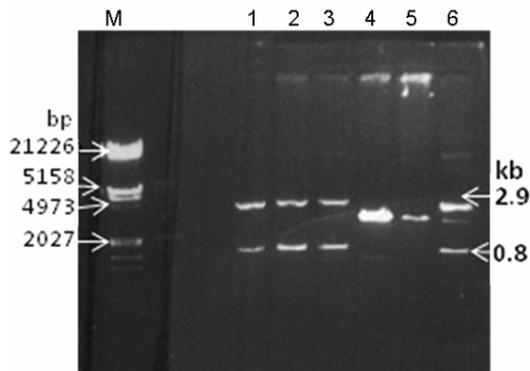


Fig. 1—Plasmid DNA of randomly selected clones after restriction with *EcoRI* and *XhoI* [Lanes 1, 2 & 6 revealed an insert of approx. 800 bp, whereas lanes 4 and 5 didn't contain any inserts; lane M, marker (lambda DNA cut with restriction enzymes *EcoRI* and *HindIII*)]

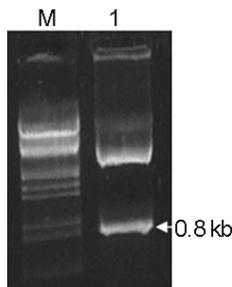


Fig. 2—1% Agarose gel showing restriction pattern of *OsZnI* plasmid with *EcoRI* and *XhoI*, [Lane 1 (an insert of 0.8 kb); and lane M (lambda DNA marker cut with restriction enzymes *EcoRI* and *HindIII*)]

having an insert size of ~0.8 kb (Fig. 2) showed the differential expression with the stressed RNA (Fig. 3a & b) and was named as pk3. The results revealed an induced expression of gene corresponding to insert of pK3 used as probe with respect to WDS.

Complete sequencing of the cDNA clone revealed an insert of 800 base pair in length (Fig. 4). The pk3 was characterized for its amino acid sequence using ExPASy-Translate tool. The nucleotide sequences were translated in its all six frames and 5'3' frame 1 of positive strand showed longest open-reading-frame (ORF) compared to other frames. ORF had a length of 663 bp encoding 221 amino acid residues with starting codon positioned at 82 bp and stop codon at 745 bp (Fig. 5). The sequence also contained 81 nucleotide 5'-UTR region and 53 base long 3'-UTR. There was a poly (A) tail of 20 nucleotides at the 780 position of the cDNA insert. The nucleotide composition of cDNA was 138 A, 256 G, 262 C and 142 T with G + C = 64.75% and A + T = 35.25%.

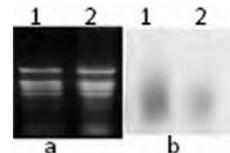


Fig. 3—(a): Agarose gel (1%) showing total RNA isolated from stressed leaves (lane 1) and from control (lane 2); and (b) Autoradiograph of gel (a) after Northern blot analysis using [³²P] labeled insert of pK3 as probe showing differential expression with stressed RNA

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GCACGAGAAGTCGCGTCGCACCCGAAGCAGAGTAGAGAAGGAGAGCGGA
GGCCATCAGCCGTCGATCGAGTATCCGATGGCTCTCGCTCTCCACC
TCTGCTGTCTGCCGCTGCCGCCGGCGGGCGCTCGCGCGAGGG
TGACGACGACGAGGGGTTACGCCGCTCGCGGGCGTCTCGCGATGA
GGCGGGCGGGCGCGGTGGAAGGGAAGGGCGCTGCGGGGATGACGC
AGCGAAGACGGGTCGTCGTCGTCGCGCGGCGAGGGAGTGTCTGTTGGT
GCCGACCCCGTGACGGGCCACTACCGCCCTCCAACCTCGCCGGGGCG
CCGACGCGCGACCTCCGCCCGCCACCTCGCCCGGCTACGCCCGCG
CGTGATGCGCGGACCTCGCGTCTCGACGGACGACGACGCCATCCA
TCCATATATATCTGCTCGCGTCAATACTCATCACTCTCGACCCGCGCG
CGCCGACGCCGCTCGCCTCCGCTGACCTGAACGACGACGACGACGC
ATCATCGCCGGGGCGGGATCGCCCGGGCGGGCGGGCGGCATCCCTAC
CGTTTCTGTCTCGGTGTGGGCTGGATGCTCTGCTGCTGTCTGTTCTTC
GTGCACCTCATGTAACAGCGAGCAGGGCCTCACTACTACTACTGTT
TTTGTTTTTTAACTTTACCGTTTTACCACGTACCGAGTGTGATGGTTTA
ATGGAACAAGTAATAAATCAGTTAATTTCTTCAAAAAAAAAAAAAAAAAA
AA
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Fig. 4—Nucleotide sequence of gene *OsZnI* (Genbank Accession no. EU503083) [Bold letters indicate coding sequence starting from ATG to TAA. Normal letters show 81 nt 5'-UTR and 53 nt 3'-UTR regions]

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MALALSTSA AAAAAAAAAAARALAA RVTTTRGYAASAASSA
MRRAAAAVEGKGAAGMTQAKD GSSSSAAREVSWVPDPV
TGHYRPSNFAGGADAPTSAPPTSPAATPARDARDLAVVR
RTTTPSIHIYLLRVNYSSPLDRAAPHAAPPPRAPERRRRRI
IARRIRRRRRHPYRFLAACGLDALLVCWFFVHLSNSE
QGLTLLLLLFLFFNFHGF TTYPSVMV
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Fig. 5—Amino acid sequence encoded by longest ORF of *OsZnI* gene

The nucleotide sequence homology search for the cDNA was done by BLASTN program using the nucleotide as query sequence in the rice data base. The BLASTN search showed homology (98-97%) with *O. sativa* Zn inducible proteins, “Maliyang (complete CDS of subsp. *indica*, Accession no. AF022734.1), “Ramy1” (subsp. *Japonica*, Accession no. AY072712.1) and “RezA” (subsp. *Japonica*, Accession no. U46138.1). This insert was named *OsZnI* and the sequence was submitted to gene bank (Accession no. EU503083). An advanced BLASTX search was carried out using amino acid sequences of *OsZnI* which also showed significant homology with Zn-inducible protein from *O. sativa* (Accession no. AAB82137.1) with common name “Maliyang protein”, *O. sativa* “RezA protein” (Accession no. AAL8605.1) and “Ramy1 protein” (Accession no. AAA87049.1). It also showed homology to LEA proteins, but homologous region extended only to N-terminal region.

Ramy1, a Zn-inducible TF has three distinct consensus sequences, a C3H Zn finger motif CXCX4CX2H, a highly conserved motif of 12 amino acids with consensus sequence WAPDPVTGYRYP homologous to LEA motif and nuclear localization signal (NLS) sequence RRRPPRRP¹⁰. The C3H Zn finger motif is important in its binding to the *cis*-acting sequence domain of regulatory region of gene promoter. *OsZnI* also showed similar conserved domains (Fig.9).

Amino acid profile and physio-chemical characterization of *OsZnI*

The restriction map of the *OsZnI* gene sequence was studied using NEB CUTTER Software with commonly used restriction enzymes. The sequence did not contain commonly used restriction enzymes such as *EcoRI*, *XhoI*, *HindIII*, *BamHI* and *XbaI*. *OsZnI* protein was characterized for its amino acid

composition using BIOEDIT SOFTWARE. It had the highest percentage of Ala (20), followed by Arg (11), Ser, Thr, Cys and His. The polypeptide had 42.27 aliphatic, 7.73 aromatic, 15.91 basic and 7.73% acidic amino acids. Total number of negatively charged residues (Asp + Glu) was 11, whereas number of positively-charged residues (Arg + Lys) was 29. Therefore, net charge on the protein was positive. This also supported the binding ability of the protein with the DNA. The physio-chemical characterization of polypeptide was done using Proto-Scale analysis software provided by ExPASy-Translate tool. The molecular weight of polypeptide was predicted to be 23.43 kDa with a theoretical pI of 11.48.

Functional conserved domains search

A functional conserved domain search was carried out using tool CDART provided by NCBI, USA. *OsZnI* protein had one conserved functional domain between 54 and 85 aa and showed homology with LEA-3 domain, which is thought to protect against water stress in plants. The conserved domain also showed similarity with LEA5 proteins from *Glycine max*, *Nicotiana tabacum* and *Gossypium hirsutum* etc. The multiple clustal W alignment of *OsZnI* protein with the LEA group proteins showed highly conserved motif of 12 amino acids (Fig. 6). Multiple clustal W alignment of *OsZnI* protein against Ramy1, RezA and Maliyang proteins showed highly conserved motif at N-terminal end of 12 amino acids with the consensus sequence WAPDPVTGYRYP at 71-82 amino acid position in “RezA protein”, 72-83 amino acid position in “Ramy1 protein”, 71-82 amino acid position in “Maliyang protein” and 71-82 amino acid position in “*OsZnI* protein.

The phylogenetic tree analysis of protein also showed subsp. *indica* “Maliyang” protein was more close to *OsZnI* and belonged to one clan, whereas RezA and Ramy1 protein to separate clan (Fig. 7).

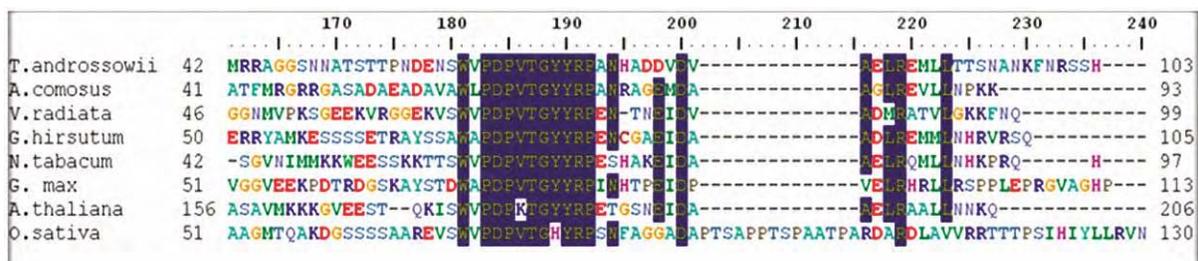


Fig. 6—Consensus sequence of LEA-3 conserved domain [Alignment of the conserved domain of *OsZnI* (*O. sativa*) with LEA3 and LEA5 protein from *Tamarix androssowii* (Accession no. AP000570), *Ananas comosus* (Accession no. AAM28284), *Vigna radiata* (Accession no. P32292) *Gossypium hirsutum* (Accession no. P46521), *Nicotiana tabacum* (Accession no. AAC06242), *Glycine max* (Accession no. 024422) and *Arabidopsis thaliana* (Accession no. 081295)]

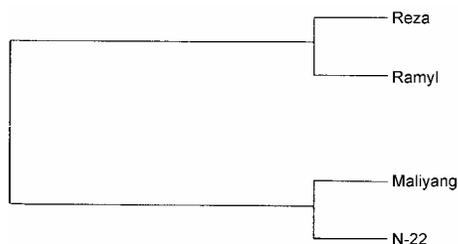


Fig. 7—Phylogenetic analysis of OsZnI protein with related proteins [*OsZnI* from N-22 (Accession no. EU503083) is compared with the Zn-inducible proteins and Reza (Accession no. AAA87049.1), Ramyl (Accession no. AAL86015.1) and Maliyang (Accession no. AAB82137.1)]

Most LEA-5 like proteins are induced in embryos or vegetative tissues by desiccation, ABA or high osmoticum. The soybean LEA5-like cDNA accumulates in desiccating seeds from 20 to 80 days after flowering and in roots, but not in leaves of drought-stressed plants¹¹. The related gene *D-21* from *Arabidopsis* displays increased transcript accumulation in roots and leaves after drought induction, but is not detected in mature dry seeds of non-stressed *Arabidopsis* plants¹². In cotton, the LEA5 transcripts are highly induced in mature and detached leaves of water-stressed plants or in water-stressed detached leaves¹³.

Prediction of OsZnI protein targeting

The sequence of OsZnI protein was further analyzed for its sub-cellular localization using software Predict NLS: Determination of Nuclear Localization in <http://123genomic.com> database. A putative nuclear localization signal sequence (RRRIAGRRIGRRRRR) was found in OsZnI protein (Arg152-Arg168) (Fig. 8). The NLS of protein lies in the N-terminal end with the sequence rich in Arg, Ile and Gly. NLS sequences were also analyzed in other homologous proteins. Rez A protein had a NLS sequence RRRPPRRP at Arg89-Pro97, Maliyang with RRRIIAARRIGRRRRR at Arg152-Arg168 and Ramyl having RRRPPRRP at Arg90-Arg98. The presence of NLS sequence suggested that OsZnI I may be transported into the nucleus through the nuclear pore complex using its own NLS. Statistical data for NLS present in the input sequence showed 97.92% NLS. These data strongly supported the protein to be nuclear localized. The classical NLSs share certain characteristics, as they generally contain several residues of Arg and Lys and may also be having residues, such as Pro that disrupt helical

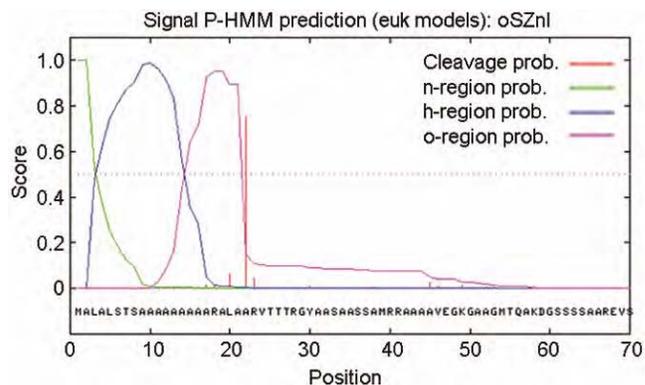


Fig. 8—Prediction of nuclear signal peptide using SignalP-HMM software

domain¹⁴. The protein was also analyzed for the signal protein using Signal P Software. Analysis showed a 21aa long signal sequence at the N-terminal end. The cleavage site probably lies between position 21 and 22: Ala-Arg.

Zinc finger domain

The zinc finger domain enables different proteins to interact with or bind DNA, RNA or other proteins and is present in the proteomes of many different organisms. Proteins containing zinc finger domain(s) have been found to play important roles in eukaryotic cells regulating different signal transduction pathways and controlling processes, such as development, programmed cell death and environmental stresses¹⁵. They are mostly plant-specific and are classified according to the number and order of the Cys and His residues that bind the Zn²⁺.

The genome of *O. sativa* codes for 189 C2H2 zinc finger TFs, which possess two main types of zinc-fingers named C and Q¹⁶. The Q-type zinc fingers contain a conserved motif QALGGH and are plant-specific¹⁵, whereas C-type are found in other organisms as well. There are many types of zinc finger proteins (ZFP), classified according to the number and order of the Cys and His residues that bind the Zn²⁺. Plant-specific Q-type C2H2-type zinc finger proteins were also searched for the 12 amino acids conserved motif (WAPDPVTGYRYP) present across the LEA group of proteins, but it did not show any such conserved domain. The C-terminal end of RAMY has a motif containing Cys and His residues reminiscent of a novel C3H zinc finger motif CXCX4CX2H and is important in its binding activity to the O2S domain of the regulatory region of the Amy2 gene promoter¹⁰. RezA protein has a similar

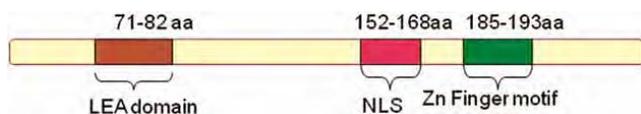


Fig. 9—Conserved domains of protein encoded by *OsZnI*

C3H motif, three Cys and one His residues CXCX4CX2H. Similar to these proteins, *OsZnI* had three Cys and one His residues containing C3H zinc finger motif CX7CX4 H X2C at the C-terminal end which is important in its binding to *cis*-acting sequence domain of regulatory region (Fig. 9).

A cDNA clone for *ZFP182* encoding a C2H2-type ZFP markedly induced in the seedlings by cold (4°C), 150 mM NaCl and 0.1 mM ABA treatments. Overexpression of ZFP 182 in transgenic tobacco and rice is shown to increase plant tolerance to salt stress¹⁷. Similarly, ZFP gene *PSTZ* has been isolated from *Populus euphratica* and has shown induced expression under drought and salt stress conditions¹⁸. TFIIIA-type of novel potato C2H2 ZFPs gene “*StZFP1*” is also involved in response to salt and dehydration stresses through an ABA-dependent pathway¹⁹. Very recently, the C2/H2-type gene *ZFP179* encoding a 17.95 kDa protein from rice has been isolated and functionally characterized against salt and oxidative stress²⁰. The expression of *Zat12* and *Zat7* ZFPs is simultaneously elevated in cells in response to oxidative stress, heat shock or wounding. *Zat12* responds to a large number of biotic and abiotic stresses and is thought to be involved in cold and oxidative stress signaling in *Arabidopsis*²¹. The four different ZPT2 related Cys2/His2-type ZFP in *Arabidopsis* have been functionally characterized and are found to be strongly induced under dehydration, high salt, cold and ABA treatments²².

Conclusion

OsZnI protein had three distinct features. NLS present in Arg152-Arg168, Zn finger domain between 185-193 amino acids and 12 amino acid conserved domain in 71-82 amino acids homologous to LEA motif and belonged to C-type family of Zn finger protein. The N-terminal end of the protein (1-110 aa) was hydrophilic in nature, while the C-terminal end (110-221 aa) was hydrophobic in nature. Further,

functional characterization of *OsZnI* may be done by transforming the cDNA in an *indica* rice variety.

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