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Cloning and heterologous expression of *Os-AP2/ERF-N22* drought inducible rice transcription factor in *E. coli*

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ABSTRACT

AP2/ERF a plant specific transcription factor plays a crucial role in the expression and regulation of abiotic stress related genes. In this study, we have characterized a rice transcription factor named *Os*-AP2/ERF-N22. It contains single AP2 domain which spans from 5th to 70th amino acid residue in the protein which is 243 amino acid residue long. The partial sequence of the gene encoding *Os*-AP2/ERF transcription factor was amplified and cloned into pET29a bacterial expression vector. The histidine-tagged truncated *Os*-AP2/ERF-N22 protein was expressed in BL21(DE3) strain of *E. coli* after induction with 0.5mM IPTG. A time course induction study was used to optimize the protein expression and the recombinant *Os*-AP2/ERF-N22 was purified using Ni-NTA agarose column chromatography. The 26.74 kDa recombinant *Os*-AP2/ERF-N22 was visualized using SDS-PAGE analysis and its expression was further confirmed by western blot analysis using anti-his primary antibody and alkaline phosphatase conjugated secondary antibody. Antibody against the purified truncated AP2/ERF-N22 was custom synthesized and it was used to quantitate the expression of *Os*-AP2/ERF-N22 transcription factor in rice at the protein level.

Key words: Abiotic stress, AP2/ERF, Drought, Heterologous expression, Rice, Transcription factor

Higher plants are challenged by various biotic as well as abiotic stresses. Biotic stresses include microbial pathogens and herbivorous insects while abiotic stresses include cold, heat, drought and salinity (Yamaguchi-Shinozaki and Shinozaki 2006). All these stresses negatively impact plant growth and development and ultimately reduce the crop yield. In order to survive and grow under such unfavourable environments the plants respond by expressing their stress responsive genes which in turn are regulated by transcription factors (Guo *et al.* 2016, Rashid 2012). The AP2/ERF (APETELA2/ethylene-responsive element binding factor) family is a large group of plant-specific transcription factors containing AP2/ERF DNA binding domain that includes four major subfamilies of transcription factors: AP2, RAV, ERF and DREB (dehydration-responsive element-binding protein) subfamilies (Sakuma *et al.* 2002). Members of the AP2 (APETELA2) subfamily contain two AP2/ERF domains connected by a conserved linker of 25 amino acids. Members of RAV (Related to ABI3/VP1) subfamily contain two different conserved DNA binding domains: AP2/ERF domain and B3 domain. However, the members of DREB, ERF and other subfamily contain a single AP2/ERF domain (Saleh and Pages 2003). This domain was first reported in *Arabidopsis* homeotic gene *APETELA2* (Jofuku *et al.*1994). AP2/ERF family members are encoded by 170 genes in rice (Rashid *et al.* 2012), 145 genes in *Arabidopsis* (Sakuma *et al.* 2002), 200 genes in poplar (Zhuang *et al.* 2008), 291 genes in Chinese cabbage (Song *et al.* 2013), 116 genes in Moso bamboo (Wu *et al.* 2015), 171 in foxtail millet genes (Lata *et al.* 2014) and 178 genes in sorghum (Srivastav *et al.* 2010).

Across the world rice is one of the most important food crops and is very sensitive to water deficit stress (Kim *et al.* 2015). Drought stress reduces the growth and severely affects traits, such as photosynthesis, stomatal conductance, starch metabolism, seedling biomass and plant water relationship (Ahmadikhah and Marufinia 2016). Grain yield of some rice varieties was reduced by up to 81% under water deficit condition and this reduction depends on timing, duration and severity of drought stress (Pantuwan *et al.* 2000).

Based on the use of semi quantitative PCR Mawlong *et*

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al. (2014) reported that the expression of the gene encoding Os-AP2/ERF-N22 transcription factor increased at transcript level under drought. Further functional validation of stress responsive *Os-AP2/ERF-N22* was studied in *Arabidopsis*. Transgenic *Arabidopsis* plants showed improved turgor and less wilting compared to wild type under water-stress; however, some phenotypic aberrations were observed (Mawlong *et al.* 2015).

Therefore, the study of this transcription factor is predicted to be a major leap towards the development of drought tolerant variety of rice. To develop such understanding of Os-AP2/ERF-N22 transcription factor the study of its biophysical property is very much essential. Further induced *Os-AP2/ERF-N22* expression under drought needs to be substantiated at protein level too. Since expression of transcription factors is temporal under control of specific cellular signals and they are usually expressed in amounts that is insufficient to purify them by conventional means. Therefore, in order to meet the above objectives, *Os-AP2/ERF-N22* encoded protein product was purified in a heterologous system such as *E.coli*. The *E.coli* is still the organism of choice for heterologous expression of foreign genes due to its fast multiplication and ease in culturing it under laboratory conditions. Therefore, the aim of this study was to clone the gene encoding Os-AP2/ERF-N22 transcription factor in pET29a expression vector, its heterologous expression in *E. coli* and its purification using Ni-NTA affinity chromatography. Further, the antibody generated against this truncated protein was used to determine the expression of Os-AP2/ERF-N22 transcription factor at protein level in rice.

MATERIALS AND METHODS

Seeds of rice (*Oryza sativa* L. cv. N-22) were germinated at room temperature. The seedlings were then transplanted into pots filled with soil and cowdung (2:1 ratio) in the net house at Division of Biochemistry, IARI, New Delhi. Leaves of 40 days old seedlings were used for this experiment. *E. coli* strains DH5α and BL21 (DE3) were purchased from New England Biolabs (England). pGEMT Easy vector was purchased from Promega (USA) and pET29a vector from Novagen (Germany). We purchased chemicals from the following sources: isoprophl-β-D thiogalactopyranoside (IPTG), dithiothreitol, and Commassie Brilliant Blue R250 from Sigma (USA); SDS, agarose, acrylamide, bisacrylamide, Taq PLUS DNA poymerase and Ni-NTA columns from G-BIOSCIENCES (USA); Phusion High-Fidelity DNA polymerase and all restriction enzymes from New England Biolabs (England); Luria-Bertani Broth and Luria-Bertani Agra from Hi-Media (India); TRI reagent, T4 DNA Ligase from Thermo Fisher scientific (USA); Methanol and Glacial acetic acid from Merck (Germany).

Total RNA was extracted from leaves of 40 days rice seedling using TRIZOL reagent according to the manufacturer's instruction. Subsequently the RNA was treated with DNase I to remove residual genomic DNA. Using this total RNA as template, the first strand cDNA was synthesized according to the protocol of Thermo Fisher scientific (USA) RevertAid first strand cDNA synthesis kit (Cat. No. K1622).

Os-AP2/ERF–N22 (LOC_Os06g40150.1 full-length cDNA sequence was obtained from the PHYTOZOME database. The forward primer 5'ATGGGACAGTCGAAGAAGAAGTTC3' and reverse primer 5'TCAGATGACGAGGCTACCTTCAC3'were designed using primer 3 software for amplification of *Os-AP2/ERF–N22* CDS from rice. The 50 µl reaction mixture for PCR amplification consisted of 100ng cDNA, 0.4µM of each primer, 0.2mM of dNTP (each), 1X Phusion HF buffer and 1.0 IU of Phusion polymerase. The PCR product was purified and cloned into pGEMT easy vector and sequenced.

In order to construct the recombinant expression vector pET29a-partial Os-AP2/ERF–N22, the primers were designed using prime 3 software.The N-terminus truncated coding region (excluding stop codon) of Os-AP2/ERF-N22 transcription factor was amplified with the following set of primers: Forward: 5'CGCGGATCCATGAAGAGGAGGGTGTGGCTG3' and Reverse: 5'ACGCCTCGAGGATGACGAGGCTACCTTCAC3'. The BamH1 restriction site in the forward primer and the XhoI restriction site in the reverse primers are underlined respectively. The 50 µl reaction mixture for PCR amplification consisted of 100 ng cDNA, 0.4 µM of each primer, 0.2 mM of dNTP (each), 1X Phusion HF buffer and 1.0 IU of Phusion polymerase.In order to express the truncated Os-AP2/ERF–N22 protein with a 6X histidine residue at its C terminus the PCR amplified product was ligated into Bam HI and Xho I digested pET29a vector. The recombinant pET29a-partial *Os-AP2/ERF–N22* plasmid was transformed into DH5α competent cells.Transformed colonies were cultured overnight in LB medium supplemented with 35 µg/ml kanamycin at 37°C and plasmid was isolated from them. To verify the recombinant pET29a-partial *Os-AP2/ERF-N22* plasmid, it was digested overnight with BamHI and XhoI and the restriction positive plasmids were given for sequencing. The restriction positive pET29a-partial *Os-AP2/ERF-N22* plasmid was used to transform BL21 (DE3) cells and colonies were selected on LA-kanamycin plates (35 µg/ml).

The recombinant BL21 (DE3) colonies were cultured overnight in LB medium supplemented with 35 µg/ml kanamycin at 37°C. Cultures were diluted 1:100 with fresh LB media supplemented with kanamycin (35µg/ml) and cultured at 37°C to 0.6 OD at 600 nm (OD_{600}). At this stage culture was equally divided into two conical flasks so that the BL21(DE3) cells can be induced with two different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG), i.e. 0.5mM and 1.0mM. Aliquots were removed from each flask at interval of 6 h, 9 h and overnight. The cells were pelleted by centrifugation at 13,000 rpm for 1 min, lysed by adding 4X SDS-PAGE loading dye, and vortexed. The bacterial lysate was denatured in boiling water for 5 min. It was then subjected to SDS-PAGE analysis on a 12%

resolving gel. Finally, the gels were stained with coomassie brilliant blue and destained with destaining solution.

The bacterial lysate was denatured in boiling water for 5 min. Equal amount of bacterial protein from control and induced culture was separated on 12% resolving gel and transferred to nitrocellulose membrane using transfer buffer (0.025 M Tris base, 0.15M glycine, and 20% methanol, pH 8.3) following the wet transfer method as described by Sambrook and Russel (2001). After transfer, membrane was washed twice for 10 min each with TBS buffer (0.01 M TrisCl, pH 7.5 and 0.15 M NaCl) and then incubated in 1% BSA for 1 h at room temperature. It was then washed twice for 10 min each time in TBS-Tween/Triton (TBST) buffer (0.02 M TrisCl, pH 7.5; 0.5 M NaCl; 0.05% v/v Tween-20 and 0.2% v/v Triton X-100) followed by washing with TBS buffer for 10 min at room temperature. After this the membrane was incubated with Anti-His (C-term) antibody at 1:500 dilutions with TBS buffer for 1 h at room temperature. It was again washed twice for 10 min with TBST followed by a 10 min wash with TBS buffer at room temperature. The membrane was then incubated with alkaline phosphatase conjugated secondary antibody (Goat anti mouse IgG/IgM secondary antibody, Invitrogen, Cat. No. T2192), diluted 1:1000 in TBS buffer for 1 h at room temperature.

To detect the expression of *Os*-AP2/ERF-N22 at protein level, the western blot analysis of protein isolated from rice plants (using method of Lin and Wang 2014) was done using the truncated *Os*-AP2/ERF-N22 specific primary antibody and alkaline phosphatase conjugated secondary antibody.

The membrane was then washed thrice for 10 min each with TBST buffer at room temperature following which, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) chromogenic substrate solution for alkaline phosphatase enzyme was added, and the blot was allowed to develop for 5 min. It was then rinsed three times with water for 2 min each. The developed blot was dried on a clean filter paper and stored in a ziplock bag at -20°C after capturing its image.

The fusion protein was purified from cell free extract of induced bacterial culture (0.5 mM IPTG, 9 h) by Ni^{2+}-NTA column chromatography according to the instructions of the kit supplier (Nucleo-pore His-Spin Protein Miniprep Kit). The column was packed with histidine affinity gel and the recombinant protein was eluted from the column using the elution buffer provided in the kit. The purified protein was subjected to SDS-PAGE on 12% resolving gel after which the primary antibody against *Os-AP2/ERF-N22*was custom synthesized.

RESULTS AND DISCUSSION

Cloning and sequence analysis of c-DNA of Os-AP2/ERF-N22 *transcription factor*

In the present study, the total RNA was extracted from rice leaves (Fig 1a). Using this total RNA, and specific primers in the RT-PCR reaction the *Os-AP2/ERF-N22* full-length cDNA was successfully amplified from rice. The amplified full-length cDNA was visualized on agarose gel as a single band of 732 bp (Fig 1b). The amplified full-length cDNA was then cloned in pGEMT vector and transformed in DH5α cells. The recombinant plasmid was restricted and the released insert was sequenced (Fig 1c). The sequencing result confirmed that *Os-AP2/ERF-N22* is 732 bp long, which encodes a 243-residue protein with a theoretical isoelectric point (pI) of 9.47 and molecular weight of 25.3 kDa. The Os-AP2/ERF-N22 transcription factor has a basic amino acid region (KKKFR) from position 5th to 9th amino acid which might act as a nuclear localization signal.

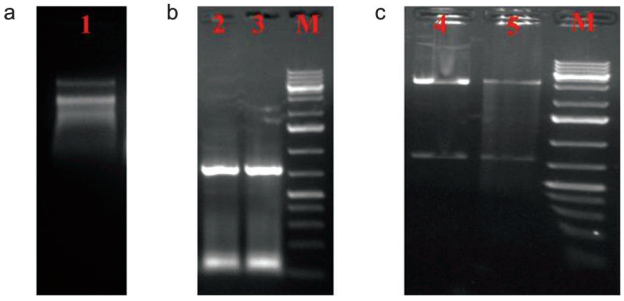

Fig 1 Cloning of cDNA of *Os-AP2/ERF-N22*. a: Agarose gel of total RNA isolated from 40 days old rice leaf. b: PCR amplification of CDS of *Os-AP2/ERF-N22* using cDNA as template. **c:** Restriction digestion analysis of *Os-AP2/ERF-N22*-pGEMT vector. M: 1kb plus gene ruler, Lane 1: Total RNA, Lane 2 and 3: PCR amplification of CDS of *Os-AP2/ERF-N22*, and Lane 4 and 5: Restriction of 732 bp *Os-AP2/ERF-N22* ORF, cloned in pGEM-T Easy Vector.

Construction of expression vector pET29a-partial Os-AP2/ERF–N22

The full length *Os-AP2/ERF-N22* is made up of two exons. The length of exon I and exon II are 83 bp and 649bp respectively. To express the *Os-AP2/ERF-N22* in *E. coli*, an efficient expression vector is essential. Initially we cloned the full-length *Os-AP2/ERF-N22* CDS into pET29a vector and tried to express the recombinant Os-AP2/ERF-N22 protein having 6X His at C terminus in *E. coli* but attempts to express full length protein product were not successful. Then, we cloned exon II (645 bp) of *Os-AP2/ERF-N22* in pET29a vector. The partial *Os-AP2/ERF-N22* digested with BamHI and XhoI was ligated to pET 29a vector digested with same enzymes. The recombinant plasmid pET29a- for *Os-AP2/ERF-N22* was transformed in DH5α cells. Preliminary verification of cloning was done by means of colony PCR (Fig 2). Plasmid was isolated from the PCR positive colonies and digested with Bam HI and Xho I (Fig 2). To express partial *Os-AP2/ERF-N22*, the recombinant pET29a-partial *Os-AP2/ERF-N22* construct was transformed into *E. coli* BL21(DE3) by conventional transformation method.

Expression of truncated Os-*AP2/ERF-N22 transcription factor in* E. Coli *BL21 (DE3) cells*

The selected BL21 cells that were successfully

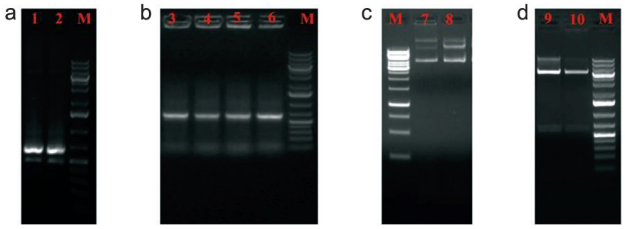

Fig 2 Cloning of partial *Os-AP2/ERF-N22* ORF excluding stop codon. a. PCR amplification of partial (Second exon) *Os-AP2/ERF-N22* ORF. b. Analysis of recombinants by colony PCR. c. Plasmid of recombinant partial *Os-AP2/ERF-N22*-pET29a vector. d. Restriction digestion analysis of partial *Os-AP2/ERF-N22*-pET29a vector vector. M:1Kb plusgene ruler, lane 1 and 2: Amplification of partial *Os-AP2/ERF-N22* ORF excluding stop codon; lane 3 to 6: colony PCR for recombinants selection; lane 7 and 8: plasmid of recombinant vector (partial *Os-AP2/ERF-N22*-pET29a vector) and lane 9 and 10: Restriction of 645bp *Os-AP2/ERF-N22* ORF, cloned in pET29a vector.

transformed with pET29a- partial *Os-AP2/ERF-N22* were cultured in LB medium along with IPTG as an inducer. The cells were harvested and subjected to SDS-PAGE analysis. The SDS-PAGE analysis which showed that the size of the recombinant protein expressed is about 26.74 kDa which is in agreement with the recombinant partial Os-AP2/ERF-N22 size (26.74) predicted by NCBI ORF finder analysis (3.67 kDa for the N- terminus vector encoded region, 22 kDa for partial *Os*-AP2/ERF-N22 and 1.07 kDa for the C-terminus 6X his residue) (Fig 3a). A time course study for optimization of induction was also carried out wherein the cells were induced with two different concentrations of IPTG (0.5mM and 1.0mM) for different time duration (6 h, 9 h and overnight) at 37°C and the subsequent SDS-PAGE analysis revealed that although fusion protein was induced under all conditions mentioned above however overnight induction with 0.5mM IPTG was found to be optimum for the expression of the recombinant protein (Fig 3a).

Western blot analysis of bacterial protein

The expression of recombinant Os-AP2/ERF-N22 having 6X his tag at its C-terminus was confirmed by western blot analysis using anti-his antibody against the histidine hexamer sequence (Fig 3b).

Purification of histidine tagged Os truncated AP2/ERF-N22 transcription factor expressed in E. coli

The truncated AP2/ERF-N22 transcription factor was freshly induced in BL21(DE3) cells by induction for 9 hr at 37°C with 0.5 mM IPTG. Subsequently his tagged fusion protein was purified from BL21(DE3) cells using Ni-NTA affinity chromatography. SDS-PAGE analysis of the purified fusion protein revealed a single protein band of 26.74 kDa (Fig 4).

Western blot analysis of plant protein

Antibody against the purified truncated AP2/ERF-N22 was custom synthesized and it was used to confirm the

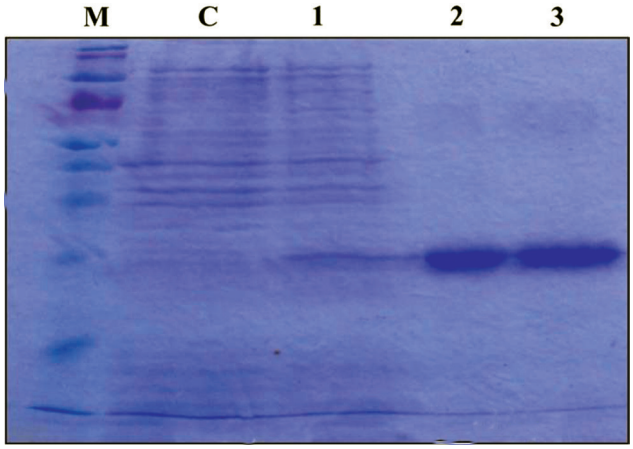

Fig 4 Purification of His tagged recombinant truncated *Os*-AP2/ERF-N22. M: Pre-stained protein ladder, C: Control, 1: lysate of BL 21(DE3) induced with 0.5 mM IPTG for 9 hr and 2 and 3: purified protein.

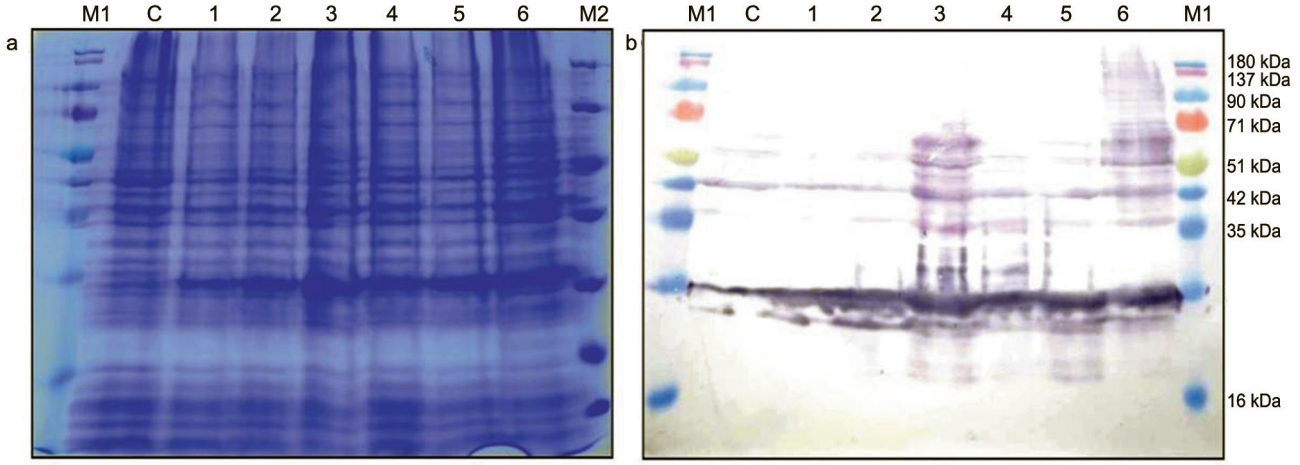

Fig 3 Expression and of His tagged recombinant truncated *Os*-AP2/ERF-N22 in *E. coli.* a. SDS-PAGE analysis of His tagged Os-AP2/ERF-N22 expressed in *E. coli*. b. Western blot analysis of His tagged *Os*-AP2/ERF-N22 in *E. coli*. M1: Pre-stained protein ladder, M2: Protein ladder, C: Control, lysate of BL 21(DE3) induced with 1: 0.5mM IPTG for 6 hrs, 2: 0.5mM IPTG for 9 hrs, 3: 0.5mM IPTG for overnight, 4: 1.0 mM IPTG for 6 hrs, 5: 1.0 mM IPTG for 9hr, 6: 1.0 mM IPTG for overnight.

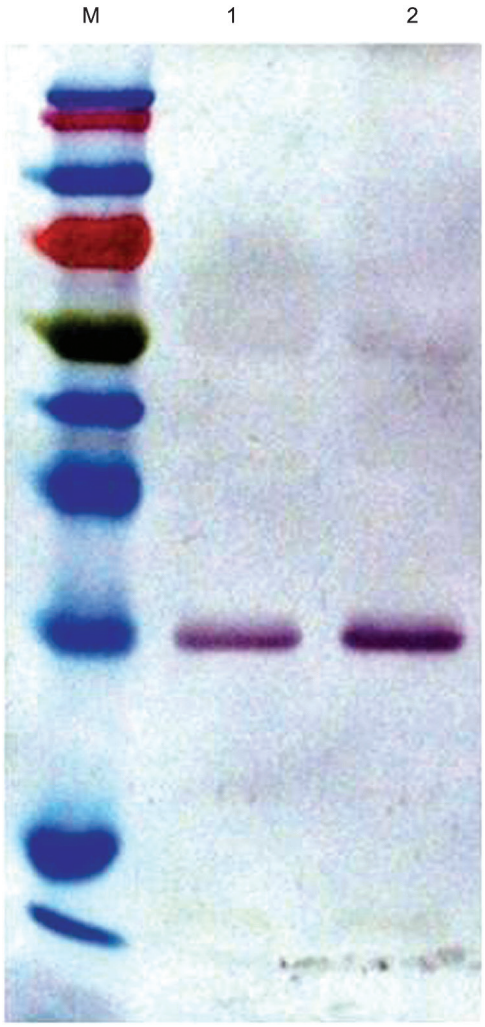

Fig 5 Western blot analysis of rice leaf protein for *Os*-AP2/ERF-N22 transcription factor using anti truncated *Os*-AP2/ERF-N22 primary antibody and alkaline phosphatase conjugated secondary antibody. M: Pre-stained protein ladder, Lane 1 and 2: bands for *Os*-AP2/ERF-N22 transcription factor protein.

expression of *Os*-AP2/ERF-N22 transcription factor in rice at protein level. The western blot analysis showed that the antibody produced against purified truncated *Os*-AP2/ERF-N22 was able to hybridize with *Os*-AP2/ERF-N22 transcription factor present in protein isolated from rice leaves (Fig 5). A protein band of approximately 25 kDa size was detected to be the *Os*-AP2/ERF-N22 transcription factor using western blot analysis. The size was found almost similar to the size of the *Os*-AP2/ERF-N22 transcription factor protein (25.3 kDa) predicted by NCBI ORF finder.

Transcription factors are master regulators of gene which control the gene expression under normal as well as stress conditions. AP2/ERF family is a large group of transcription factors which is characterized by the presence of conserved AP2/ERF DNA binding domain (Song *et al.* 2013). Os-AP2/ERF-N22 is one of the member of AP2/ERF family of transcription factors that has a single AP2 domain which spans from 5th to 70th amino acid residue (Mawlong *et al.* 2014). As a part of this study the *Os-AP2/ERF-N22* was amplified and cloned in pET29a vector. Since PCR can introduce some errors during DNA amplification (Cha and Thilly 1993), it was necessary to make sure that 732 bp amplified DNA fragment was free of any unintended mutations. To confirm the identity of amplified *Oryza sativa Indica* (N22) *Os-AP2/ERF* gene with the *Os-AP2/ERF* gene *in situ* (*Oryza sativa Japonica*), DNA sequencing was performed. Gene sequencing confirmed that the cloned DNA fragment was 100% identical to *Os-AP2/ERF in situ.*

Os-AP2/ERF-N22 protein is encoded by a 732bp ORF and its expression is reported to increase under drought (Mawlong *et al.* 2014). However very little is understood about its structural features and expression at protein level. Its over expression in a heterologous system and subsequent purification is a prerequisite to study its biophysical characteristics. In order to express this protein in amounts sufficient for its characterization we initially tried to express the full-length Os-AP2/ERF-N22 in *E. coli* but could not succeed. Although, the full-length *Os-AP2/ERF-N22* was cloned properly in pET29a vector but it could not be expressed in *E. coli* which could be either due to the instability of the complete protein in *E. coli* or due to the presence of some rare codons in exon I of this gene that cannot be recognized by the tRNAs present in the prokaryotic system. *Os*-AP2/ERF-N22 has two exonic regions, i.e. exon I and exon II. The *in silico* analysis of the *Os*-AP2/ERF-N22 sequence revealed that the epitopic region of this protein is coded by exon II of this gene and therefore the protein synthesized by the expression of this segment of the gene should be sufficient to generate antibody against this transcription factor. With this assumption, we cloned the exon II of this gene in pET29a and successfully expressed it in *E. coli*. The expression of truncated *Os*-AP2/ERF-N22 in *E.coli* was confirmed by restriction digestion and western blotting respectively. For maximum expression of the truncated *Os*-AP2/ERF-N22 in BL21(DE3) the cloned gene was transcribed under the control of bacteriophage T7 promoter which in turn is under the control of the IPTG inducible lacUV5 promoter. Time course studies for induction show that optimum induction of truncated *Os*-AP2/ERF-N22 occurs upon overnight induction with 0.5 mM IPTG. Use of C-terminus 6X His-tag has its own advantage as it facilitated the detection of the 26.74 kDa truncated recombinant *Os*-AP2/ERF-N22 protein expressed in *E.coli.*

Antibody produced against this purified truncated *Os*-AP2/ERF-N22 was able to hybridize with *Os*-AP2/ERF-N22 transcription factor present in protein isolated from rice leaves which proves that the purified truncated *Os*-AP2/ERF-N22 protein contains the epitopic region necessary for generation of antibody against it which in turn can be successfully used to test the expression of this transcription factor in rice plant.

Future work on the characterization of this protein based on the expression of this transcription factor in heterologous organisms particularly a eukaryotic host such as yeast will generate a paradigm shift in the knowledge

of this transcription factor and extend its application for inducing drought tolerance in plants.

In this study we have successfully cloned, overexpressed the 645 bp partial *Os-AP2/ERF-N22* gene in *E. coli* and purified the overexpressed protein using Ni-NTA column chromatography. The heterologous expression and purification of *Os*-AP2/ERF-N22 could be used to produce large amounts of this protein for its further biophysical characterization and antibody production against it. The antibody generated against *Os*-AP2/ERF-N22 could be used to know the expression pattern of *Os*-AP2/ERF-N22 transcription factor under drought at the protein level. This study has laid the foundation to carry out biophysical characterization related studies of *Os*-AP2/ERF-N22 which will further pave a way towards understanding the drought tolerance mechanism in rice.

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