



# Cloning and Expression Analysis of bZIP Transcription Factor from Finger Millet (*Eleusine coracana* L.) Under Abiotic Stress Conditions

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**Abstract:** Basic leucine zipper (bZIP) transcription factors comprise one of the largest gene families in plants. They play a key role in almost every aspect of plant growth and development and also in biotic and abiotic stress tolerance. In this study, we were attempted to study characterization of bZIP, a transcription factor from a climate smart cereal finger millet (*Eleusine coracana* L.). Seeds of *Eleusine coracana* (finger millet) was purchase from local market and were grown under field conditions drought and salt stress conditions. In this study, EcbZIP gene was isolated from finger millet, cloned into DH5a cells, screened by using colony PCR and expression analysis in response to two abiotic stresses was carried out by using qRT PCR. EcbZIP coding DNA sequence and protein sequence were retrieved from NCBI Nucleotide Database and Genpept of Accession number KP033192.1 and AJP67539.1 and validated by using SMART (simple modular architecture tool) Domain Tool. Cloning and expression studies were carried out using standardized molecular biology protocol. Results depicted that EcbZIP transcription factor showed significant upregulation under both salt and drought stress conditions, indicating that it plays an important role in tolerance towards these stresses. In conclusion, expression analysis of bZIP gene from finger millet seed cultivar ML-365 showed 5-fold upregulation to salt stress to drought stress and 8-fold upregulation to salt stress. Hence, it can serve as a candidate gene for improving abiotic stress tolerance and can be helpful in enhancing the crop productivity under stress conditions.

**Keywords:** Finger millet, bZIP transcription factor, Drought stress, Salt stress

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## 1. Introduction

Agricultural production and quality are adversely affected by a broad range of abiotic stresses including drought, salinity, heat, and cold. Especially when these stresses occur in combination, it can have devastating effects on plant growth and productivity. It is estimated that more than 50% of worldwide yield loss for major crop are caused by abiotic stresses.<sup>1-3</sup> According to the current climate prediction models, the deterioration of global climate will inevitably cause an increased frequency of drought, heat wave, and salinization. This means that agricultural productivity will face a greater challenge in fighting against environmental stresses. Meanwhile, the growing world population will reach close to ten billion by the year 2050 and the almost two times of current agricultural productivity is needed to feed the large population.<sup>4</sup> Moreover, such a tremendous increase of crop productivity must be achieved with no increase in arable land and in the face of multiple environmental stresses.



Many scholars and experts worldwide have reached a consensus that breeding stress-tolerant crops with higher yields and improved qualities against multiple environmental stresses is an effective strategy, as well as one of the greatest challenges faced by modern agriculture.<sup>5-7</sup> Two main approaches have been employed to this process. One is traditional breeding methods which often bring about unpredictable results. Another is modern transgenic technology by introducing novel exogenous genes or altering the expression levels of endogenous genes to improve stress tolerance. Since conventional breeding approaches have marginal success due to the complexity of stress tolerance traits, the transgenic approach is now being popularly used to develop transgenic crops tolerant to abiotic stresses.<sup>8</sup> Therefore, deciphering the molecular mechanisms by which plants perceive and transduce stress signals to cellular machinery to initiate adaptive responses is an essential prerequisite for identification of the key genes and pathways to engineer stress-tolerant crop plants.<sup>9-11</sup>

As one of the most important regulators, TFs function as terminal transducers and directly regulate the expression of an array of downstream genes by interacting with the specific cis-elements in their promoter region.<sup>12</sup> In the last few decades, considerable research has been conducted to identify and characterize various TFs involved in plant abiotic stress responses either in abscisic acid (ABA)-dependent pathway or ABA-independent pathway, three such TF families are *AP2/ERF*, *MYB* and *WRKY*.<sup>13,14</sup>

The degree of susceptibility to abiotic stresses varies from species to species.<sup>16</sup> Finger millet is a highly productive crop that can thrive under a variety of harsh environmental conditions, and is also organic by default. It can be grown on low fertility soils and is not dependent on the use of chemical fertilizers, hence, is a boon for the vast arid and semi-arid regions.<sup>17</sup> The different genotypes of finger millet have genes for early and vigorous growth, large panicle size, increased finger number and branching as well as high-density grains. Some of the genotypes are water-efficient with elevated carbon dioxide fixation rates and minimal leaf area and hence could perform extraordinary well in semi-arid climates. It is also known to be one of the most efficient utilizers of nitrogen.<sup>18</sup> Finger millet seeds can resist storage pests for as long as 10 years, ensuring round the year food supply or even during a crop failure, that has earned it the popular name of 'famine crop'.<sup>19</sup>

Though finger millet is considered to be generally abiotic stress tolerant, even then there is a need to identify newer sources of stress tolerance in this crop that can be used for crop improvement programs. Assessing patterns of genetic diversity in terms of both abiotic and biotic stress tolerance in finger millet germplasm collections could be very critical. A large number of finger millet accessions have been preserved in various national and international GenBanks in Asian, African, and European countries as well as USA. India has the largest collection of finger millet germplasm followed by Ethiopia.<sup>20</sup> There could be immense morphological and genetic diversity among finger millet accessions or their core collections. Molecular markers could be used to characterize functional diversity in this crop.<sup>21</sup> Calcium dynamics,<sup>22</sup> tryptophan accumulation and association mapping,<sup>23,24</sup> and disease resistance<sup>25</sup> have been characterized using molecular markers. However, characterization of abiotic stress tolerance in finger millet using molecular markers is yet very limited and offers an opportunity of exploring the vast collections of wild and cultivated accessions of this crop. Therefore, in the current we attempted to study characterization of bZIP, a transcription factor from a climate smart cereal finger millet (*Eleusine coracana* L.).

## 2. Materials and Methods

### 2.1 Plant germination and stress treatment

Seeds of *Eleusine coracana* (finger millet) cultivar ML-365 was purchased from local market and were grown under field conditions. After 10 days of growth saplings were transferred to quarter strength Hoaglands media in order to impose drought and salt stress. Drought stress was stimulated by withholding water and for salt stress was imposed by adding 200mM NaCl to the quarter strength Hoaglands media and grown hydroponically for 24hours. After the stress treatment, 24hours control and stress exposed tissues were harvested and stored at -80°C for further analysis.



## **2.2 Isolation and amplification of bZIP by PCR**

### **2.2.1 Isolation of total RNA**

The total RNA was isolated by modified Chomczynski method using RNA Iso plus (TAKARA).<sup>26</sup>

### **2.2.2 cDNA synthesis using total RNA**

The total RNA template was converted to complementary DNA (cDNA) by reverse transcription with total RNA using iScript cDNA Synthesis Kit for Reverse Transcriptase PCR (BIO-RAD) according to manufacturer's instructions. cDNA concentration was checked using Nanodrop2000 (ThermoScientific).

### **2.2.3. Sequence retrieval and primer designing**

EcbZIP coding DNA sequence and protein sequence were retrieved from NCBI Nucleotide Database and Genpept of Accession number KP033192.1 and AJP67539.1 and validated by using SMART (simple modular architecture tool) Domain Tool. For EcbZIP sequence primers were designed using Primer3 plus Software (<http://bioinfo.ut.ee/primer3-0.4.0/>) with the following parameters – Forward and Reverse primers of 26bp, 50-55% GC content, T<sub>m</sub> of 60.13 and 59.92 respectively. The restriction sites were inserted in both forward and reverse primers at 5' end for BamHI (GGATCC) and XhoI (CTCGAG) restriction enzymes along with 4bp sitting sequence.

## **2.3. Molecular cloning**

### **2.3.1 Competent cell preparation**

The DH5 $\alpha$  cells were grown overnight on LB plate and moderately sized single colonies of DH5 $\alpha$  were picked and inoculated into LB media followed by incubation in shaker ~150rpm at 37°C, OD at 600 was measured after every hour to get the 0.2-0.4 OD cells were immediately transferred on ice when it reached 0.4 OD and chilled the culture for 30 minutes with occasional mixing, then the cells were harvested by centrifugation at 3000xg for 15 minutes at 4°C, the pellet was gently resuspended in ice cold MgCl<sub>2</sub>[100mM] and was centrifuged at 2000Xg for 15 minutes at 4°C, then the pellet was resuspended in ice cold CaCl<sub>2</sub>[100mM] and kept on ice for 20 minutes and was again centrifuged at 2000Xg for 15 minutes at 4°C. The pellet was resuspended in small volume of ice cold 85mM CaCl<sub>2</sub>, 15% glycerol [v/v] and was centrifuged at 1000Xg for 15 min at 4°C. The pellet was resuspended in small amount of ice cold 85mM CaCl<sub>2</sub>, 15% glycerol [v/v] followed by aliquots of 50ul into sterile vials and was stored in -80°C freezer.

### **2.3.2 Plasmid isolation**

Plasmid DNA was isolated from overnight grown culture of *E. coli* harboring pET28a (+) by alkaline lysis method.<sup>27</sup>

### **2.3.3 Gel elution of bZIP and pet28 USING Gene Jet extraction kit**

DNA was extracted from the gel using centrifuge Step Procedure using Gene Jet Purification Kit according to manufacturer's instructions.

### **2.3.4 Restriction Enzyme double digestion of vector and insert**

Purified plasmid and EcbZIP PCR amplicon were subjected to restriction double digestion using BamHI and XhoI restriction enzymes simultaneously. In each case the reaction was setup as follows using 10X NEB buffer. HindIII was heat inactivated at 80°C for 20 minutes but BamHI-HF cannot be heat inactivated thus it was inactivated by adding 6X NEB purple gel loading dye. This RE double digested mixture was run on agarose gel and then eluted for Ligation.



### 2.3.5 Ligation

The restriction digested plasmid and insert (EcbZIP) were gel eluted using Gene JET purification Kit and was used for ligation in the ratio 3:1(insert: vector). The reaction was carried out using T4 DNA ligase (Thermo Scientific) and its 10X buffer at 22°C overnight. This mixture was taken for Transformation.

### 2.4. Transformation of plasmid carrying bZIP gene into dh5 $\alpha$ competent cells

Setup for transformation

Components	Vial 1	Vial 2
Competent cells	100ul	100ul
Ligation mixture	5ul	-
Control mixture	-	5ul

This mixture was incubated for 30 minutes on ice followed by a brief 45 second heat shock at 42°C and immediately placed on ice for 2 minutes. 900ul of LB broth was added and was incubated for 1hour at 37°C in shaker incubator (150 rpm). After incubation it was plated on agar plate containing Kanamycin and was incubated overnight at 37°C. (Table 2)

### 2.5. Colony PCR

From the transformed culture plate three isolated colonies were picked using a sterile tooth pick and swirled in minimal amount of distilled water taken in different vials. The cells were lysed with a short heating step. 2ul of this lysed cell solution was used for colony PCR in order to validate the presence or absence of transformed colonies. Controls used in this experiment were DH5 $\alpha$  competent cells and DH5 $\alpha$  competent cells containing pET28a (+) as negative controls in order to check the gene specific primers were complementary to bacterial genomic DNA or to the plasmid DNA. A no template control was also done as another negative control.

### 2.6. qRT PCR

To study the drought and salt expression levels of bZIP, total RNA was isolated from drought and salt stress induced tissues of finger millet genotype; and was converted to cDNA as described earlier. Using bZIP gene-specific primers, expression profiling was carried out in all the tissues. Real-time PCR (qPCR) was carried out using the 2x Brilliant III SYBR Green qPCR Master Mix, Agilent according to the manufacturer's instructions using CFX96 Real-Time System. (Table 1)

Table 1: Reaction composition of qRT PCR

Component	Volume per reaction
cDNA	1ug
2x Brilliant III SYBR Green QPCR Master Mix with High ROX	10ul
Forward Primer	1ul
Reverse Primer	1ul
Total Volume made up with nuclease free water	20ul

The CYP gene was used as an internal control, the amplification profile used for qPCR was 95 °C for 2-min initial denaturation followed by 30 cycles of 95°C for 3mins, 95 °C for 13 s, 50°C for 30 s, and 55 °C for 30 s.

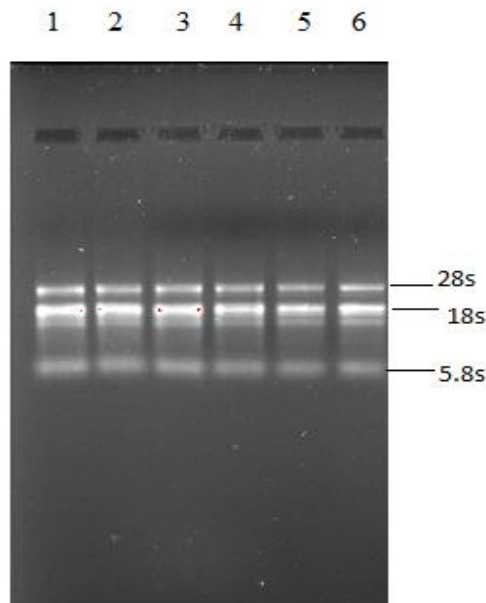


All samples were amplified in duplicates and the mean value was considered. The normalized transcript expression was quantified using the  $2^{-\Delta\Delta CT}$  method and presented as fold change over control.<sup>28</sup>

### 3. Results and Discussion

#### 3.1. Total RNA isolation

Total RNA was isolated from 10 days old saplings. The concentration of the total RNA was determined using Nanodrop 2000 and was found to be 250ug/ul. To check the quality of the total RNA before proceeding for further steps, RNA was run on 1% agarose gel which is depicted in the Figure 1. Three bands were obtained which represents 28s rRNA, 18s rRNA and 5.8s rRNA indicating the total RNA isolated was intact and had not undergone degradation. Total RNA was taken as template for the synthesis of cDNA (complementary DNA) by reverse transcriptase. The concentration of the cDNA was determined using Nanodrop 2000 and was found to be 100ug/ul (Figure 1).



**Figure 1:** Isolated RNA from Finger Millet (ML 365). Lane 1 to 6: Isolated RNA; First band from top represents 28s rRNA, second band 18s rRNA and third band 5.8s rRNA.

#### 3.2. Primer designing

The coding DNA sequence and protein sequence of EcbZIP, obtained from NCBI Nucleotide data base and Genepept were:

##### *Coding DNA sequence*

```
AATATCCCAATCGAGCTACATGGACGCGGACTTCTTCGCTGACCTCGACTTTGTGCGCCCTCCTCGCATCC
TTCTCCGGCGAGCCCGCCCGCTCTCCGATATTATCATCCCCTCCCCTCCCGCCGTCGCCCGCGGC
CGATGCGGAGGCGGGGTCGCCGGAGTCAGTGACTTCCCGTTCGAGCCCTCCGGCGGAGGCCCTATCGGA
GATCGAGAGGTTCTGATGGAGGAGGGGAGGCGGAGGCGACGGGGCGGTGGAAGGGATCAGCGTGGAG
GAATTCTTCGACGCGCTGTACGACGCGGGGAGGAGAAAGAGAAGGAGAGCGAGGCGGGCGGGAGCACGG
ATTCGGGCTCCGGCAGGGAGGAGGAGGTTGCGACGCCGGAGGCGGAGAAAGTTGGAGGTGGATGGCGATGA
CCCCGTCAGCAAGAAGAAGAGGAGGCAAATGAGGAACAGGGATTCTGCCATGAAATCGAGGGAGAGGAAG
AAGTTATATGTAAAGGAATTGGAGACGAAGAGCAAGTATTTGGAGGCAGAGTGCCGCCGTCTCAGCTACG
CACTCCAGTGCTACGAGCTGAGAACATGACACTGCGCCAGAGCTTGCTGAAGGATAGGCCTGTTGGTGC
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TCCCACAGCCATGCAGGAGTCTGCCGTACTCACGGAAACCCCTGCCGATGGTTTCCCTGCTTTGGCTGGTG  
AGCATCGTGTGCCTATTCCCTAATGCCCGGTCTACCCAACCAAGCTCAGCTGCTCCAAGAAGCAGCGGGA  
GAGATCTCGTGACGGTAGCCGGAAAGCCAAGCAGTGAAGAACCCAGAGACACTGGACCTCATCCTCCATGG  
AAGGCGTTGCAAGGGCACAAGGGCGAAGATCAAGCTATCTACCTTACCGTTTCATGCTGCAGCAGCTTGT  
TAGGCTAGGCTTATCATGATCTGTAGTAGTCGCCTTTCCAAGGCTTAGCATGCCTATGGCATGTGCATCC  
ATGGAGTATGTACCTATTTTTTTTTGTTCTTTTTTCTCTCTGATTTATGATCCCATCGTACCCAGAGTAGT  
TTTTCAATCTGGTTTGTGTTATGTCTTTTCGTCTCGCTGCTCCAGGATCGAAGAATAGTCGGGACCAAAA  
CAGTGAAGTATGAAGCTTGGACCTATAGTCATCTATAAAATCTTCGATTATATGCAAAAAAAAA
```

### Protein sequence

>AJP67539.1 bZIP transcription factor [Eleusine coracana]  
MDADFFADLDFVALLASFSGEPAAVSDIIPSPPPSPPARDAEAGSPESVTSRSPPAEALSEIERFLM  
EEGEAEGDGAVEGISVEEFFDALYDGGEEKEKESEAGGSTDSGSGREEVATPEAEKLEVDGDDPVSKKK  
RRQMRNDSAMKSREKLYVKELETKSKYLEAECRRLSYALQCYAENMTLRQSLKDRPVGAPTAMQE  
SAVLTELEPMVSLWLVSVIVCLFLMPGLPNPSSAAPRSSGRDLVTVAGKPSSENPETLDLILHGRCKGT  
RAKIKLSTLPFHAAAAC

### Validation of Protein sequences using SMART Domain Tool

Domains within *Eleusine coracana* protein **A0A0C5KQY6\_ELEC0** (A0A0C5KQY6)

E2F transcription factor

Length: 297 aa  
Source database: UniProt  
Identifiers: A0A0C5KQY6\_ELEC0, A0A0C5KQY6

The SMART diagram above represents a summary of the results shown below. Domains with scores less significant than established cutoffs are not shown in the diagram. Features are also not shown when two or more occupy the same piece of sequence; the priority for display is given by SMART > PFAM > PROSPERO repeats > Signal peptide > Transmembrane > Coiled coil > Unstructured regions > Low complexity. In either case, features not shown in the above diagram are marked as 'overlap' in the right side table below.

Confidently predicted domains, repeats, motifs and features:			
Name	Start	End	E-value
low complexity	2	18	N/A
low complexity	22	40	N/A
low complexity	71	83	N/A
low complexity	96	109	N/A
BRLZ	133	198	0.00000177

Features NOT shown in the diagram:

There are no hidden domains or features present.



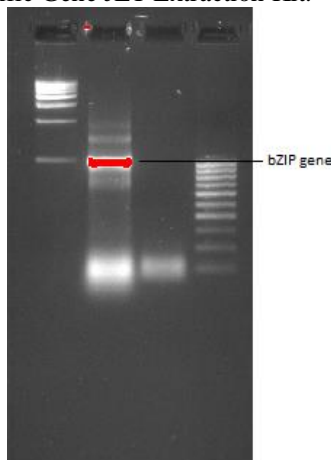
Using primer3 plus software specific primers for EcbZIP were design:

**Table 2:** Primer for EcbZIP

Forward PRIMER having BamHI restriction site	5'atatGGATCCCCGTCAGCAAGAAGAAGAGG 3'
Reverse PRIMER having XhoI restriction site	5'atatCTCGAGGGGCATTAGGAATAGGCACA 3'

### 3.3. PCR amplification

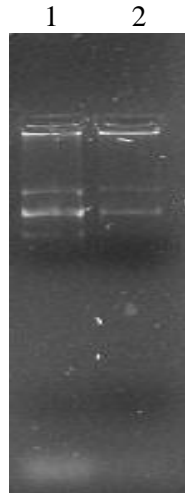
In order to amplify bZIP gene, cDNA was used as template and PCR reaction was set up with gene specific forward and reverse primers along with an NTC. The amplified mixture was run on 1.5% agarose gel along with 100bp and 1kb ladder (Figure 2). A single band was obtained. (Figure 2) The amplified mixture was eluted from gel by using ThermoScientific Gene JET Extraction Kit.



**Figure 2:** Amplified bZIP gene. Lane 1: 1kb ladder; Lane 2: bZIP gene, Lane 3 NTC; Lane 4: 100bp ladder

### 3.4. Plasmid isolation

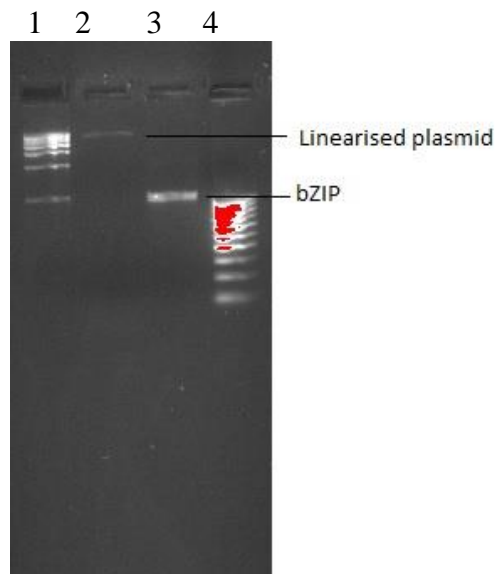
To clone bZIP gene, pET28a plasmid was isolated by alkali lysis method and purified by phenol chloroform isoamyl alcohol. After isolation, the dissolved plasmid was given RNase, a treatment to remove RNA. The quality of the plasmid was determined by agarose gel electrophoresis. The plasmid was resolved into 3 bands representing linear, circular and supercoiled DNA (Figure 3). The concentration of purified plasmid determined by Nanodrop was found to be 200ug/ul.



**Figure 3:** Pet28a plasmid on 1% agarose gel. Lane 1 and 2: pET28a plasmid DNA showing linearised, circular and supercoiled DNA bands

### 3.5. Restriction digestion of purified plasmid and EcbZIP gene

The purified plasmid and bZIP gene were digested with Xho1 and BamH1 to create cohesive ends. The digested plasmid mixture was run on 1% agarose gel to check for complete digestion. The plasmid was linearized and showed single band of 5.3kbp (Figure 4).



**Figure 4:** Restriction digestion of pET28a and bZIP for Ligation; Lane 1; 1 kb ladder; Lane 2; Linearised plasmid; Lane3; Linearised bZIP; Lane 4; 100bp Ladder





### 3.6. Cloning of EcbZIP into DH5 $\alpha$ cells

The linearized plasmid and the digested bZIP gene were ligated using T4 DNA ligase and transformed into *E. coli* host DH5 $\alpha$ . The transformed culture was plated on kanamycin containing LB agar and incubated at 37 °C overnight and figure 5 shows the positive control.

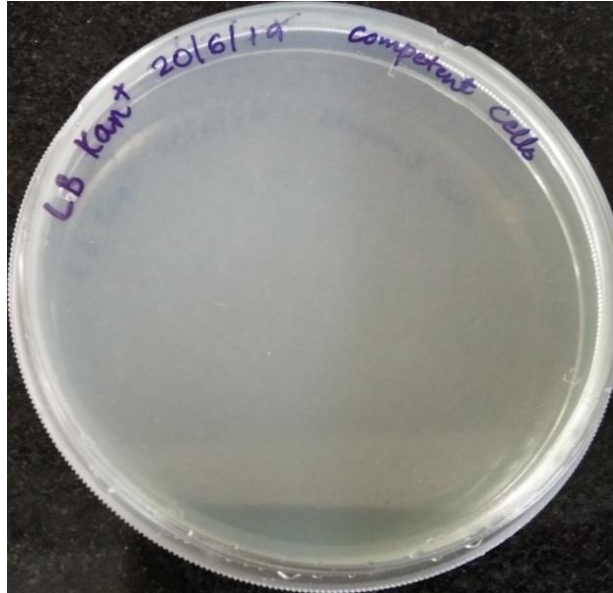


**Figure 5:** Positive control: The transformed colonies after cloning on LB agar plate containing Kanamycin

To check the efficiency of competent cells it was grown on LB agar plate containing Kanamycin. The competent cells were plated on LB agar in the presence and absence of kanamycin and was incubated overnight at 37<sup>o</sup> C as shown in figure 6 and 7 respectively.



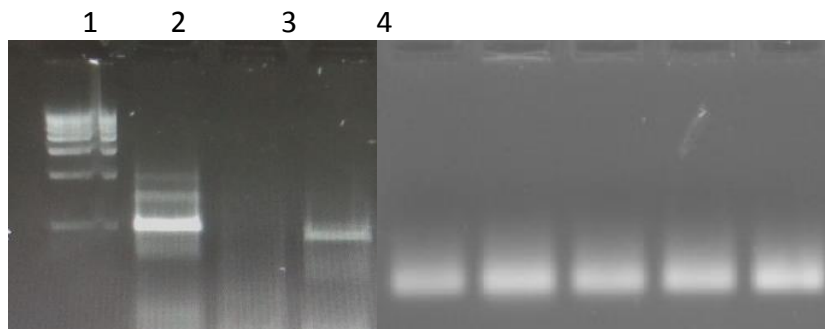
**Figure 6 :** Competent cells grown without Kanamycin



**Figure 7:** Competent cells grown with Kanamycin

### 3.7. Screening of recombinant plasmids by colony PCR

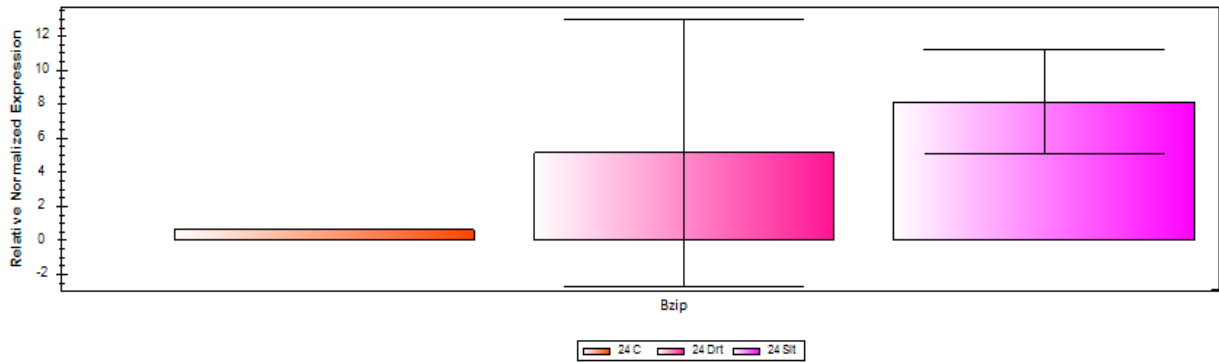
Colony PCR is a convenient high through put method for determining the presence and absence of insert DNA in Plasmid grown up on antibiotic containing media following transformation, by using gene specific primers. Colony PCR was carried out by picking three colonies from transformed culture plate and run on agarose gel to check for the bZIP amplicon. Gel was visualized using BioRad gel documentation unit. Out of three colonies picked in this experiment two were confirmed to contain insert (Figure 8).



**Figure 8:** Colony PCR Gel; Lane 1; 100bp Ladder; Lane 2 and 4 showing positive clones; Lane3 negative clone

### 3.8. Differential expression analysis of bZIP

The expression pattern of EcbZIP under drought and salt stress was investigated by qPCR. EcbZIP showed a 5-fold upregulation under drought and an 8-fold upregulation under salt stress. It was found to be up regulated in both drought and salt stress (Figure 9 and Table 3).



**Figure 9:** Relative normalised expression of EcbZIP under salt and drought conditions

**Table 3:** Quantification data showing expression of bZIP under salt and drought stress conditions

Target	Sample	Expression	Expression SEM	Mean Cq
CYP	24C	N/A	N/A	27.94
CYP	24Drt	N/A	N/A	30.44
CYP	24SlT	N/A	N/A	30.97
bZIP	24C	0.62850	0.00000	35.12
bZIP	24Drt	5.14503	7.82143	34.58
bZIP	24SlT	8.14513	3.07544	35.98

Transcription factors of the bZIP family control important processes in all eukaryotes. In plants, bZIPs are regulators of many central developmental and physiological processes including abiotic and biotic stress responses.<sup>2</sup> bZIP TFs have been reported to be excellent candidate genes for the improvement of stress tolerance in crop plants,<sup>24</sup> and several bZIP transcription factor genes have been cloned from *Arabidopsis*, rice, soybean, maize etc., and found to participate in plant abiotic stress-response.<sup>4,23,25</sup> In line with these results, we carried the expression profiling of EcbZIP under abiotic stress and found that the EcbZIP transcription factor showed significant upregulation under both salt and drought stress conditions, indicating that it plays an important role in tolerance towards these stresses.

#### 4. Conclusion

In conclusion, expression analysis of bZIP gene from finger millet seed cultivar ML-365 showed 5-fold upregulation to salt stress to drought stress and 8-fold upregulation to salt stress. Hence, it can serve as a candidate gene for improving abiotic stress tolerance and can be helpful in enhancing the crop productivity under stress conditions.



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