



## Selection of reliable reference genes for quantitative real -time PCR of salt responsive genes in three kodo millet (*Paspalum scrobiculatum*) accessions against Abiotic Stress conditions

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### Abstract

Kodo millet is the hardiest crop among the small millets. The crop harvested as a cereal in West Africa and in India. Kodo millet is ability to tolerate the biotic and abiotic stresses. The crop is subjected to salinity stress at germination, flowering and grain filling stages which leads to the low yields. Therefore, the best option for crop production, yield improvement under salinity stress is to develop stress tolerant crop varieties. It is important to study the response of Kodo millet varieties at molecular level for better development of salinity tolerant lines.

Quantitative real-time polymerase chain reaction (RT-qPCR) using a stable reference gene is widely used for gene expression research. For any successful molecular analysis especially for qRT-PCR studies, the extraction of pure RNA is mandatory. we have used LiCl<sub>2</sub> method through which we produced pure RNA for gene expression studies. A total of 19 salt stress responsive gene primers are used.

Among all the test accessions IC – 426676 reported high expression levels of all the genes tested which correlates with their existence against salt stress. Whereas, the accession IPS 351 showed moderately tolerant towards saline stress condition. But the accession IPS 145 recorded very poor expression levels which correlate with its phenotypic existence. Hence this accession can be recommended as suitable to grow under salt stress conditions. Further, IC 426676 germplasm has a scope to exploit them in future plant breeding and transgenic experiments to improve salt tolerant kodo millet germplasm.

**Keywords:** Kodo millet, NaCl stress, RT-PCR, Accessions, RNA, lithium chloride

### Introduction

Kodo millet is one of the small grain cereals with cultivation history of more than 3,000 years in Indian sub-continent. *Paspalum scrobiculatum* commonly called as kodo millet belongs to family Poaceae of tribe Paniceae. It has great importance in the Deccan plateau and particularly important in the semi-arid subtropical regions of Asia and Africa (Adhikari 2010). It grows on a fairly large scale for food and livestock feed in India and Africa.

Gene regulation in eukaryotes is a critical aspect leading to normal development, energy conservation and metabolic control organisms. In general, plants are affected by more than one stress at a particular point of time. Salinity is one of the important abiotic factors and it results due to accumulation of soluble salts under water logged conditions. The problem of salt stress turn into a major concern when formerly fertile, productive agricultural lands are salinized more intensely as a result of anthropogenic activities along with normal causes. (Arora 2019) [3]. The complex regulatory mechanism involved in the expression of genes requires highly sensitive and accurate instrumentation that quantifies the differentially expressed gene(s) under varying experimental conditions.

Quantitative Real-time PCR (RT-qPCR) is an lively tool used to analyse the expression of target genes in different tissues and organs (Busti *et al.* 2005 [7] and Artico *et al.* 2010) [4]. It has many advantages over conventional techniques like microarray and Northern blot in terms of rapidity, sensitivity, and reproducibility in synchronized quantification of transcriptional abundance in different samples for a number of genes at a time (Ginzinger *et al.* 2002 [10] and Kubista *et al.* 2006) [18]. The RT-qPCR offers specific quantification of the gene expression changes, it is

easily pretentious by certain factors, such as quality (RNA integrity) and quantity (concentration) of RNA samples, efficiency of enzymes (DNA polymerase/reverse transcriptase), the specificity and efficiency of primers and the overall transcriptional activity of the cells analyzed (Kumar 2013) [19].

In RT-qPCR analysis, the use of internal control or reference gene is the common strategy that is being widely functional across biological systems (Guénin *et al.* 2009 [11] and Schmidt *et al.* 2010). Real-time RT-PCR is the recommended method for quantitative gene expression analysis. The Plants of Kodo millet release high levels of polyphenols, polysaccharides and RNase in times of stress that make RNA extraction often tough (Kansal *et al.* 2008).

mRNA is the main target of real-time PCR analysis; hence, it would be more particular to consider the quality of mRNA directly. Moreover, the assessed quality of the extracted RNA used for real-time PCR may be more related using PCR-based procedures (Vermeulen *et al.* 2016) [33]. The selection of an individual RNA extraction technique can work more successfully with one particular tissue and can also affect the total RNA yield up to 10-fold (Pfaffl MW 2004).

Stress responsive genes can be generally categorized into two groups' viz., functional proteins and regulatory proteins based on their encoding products. Functional proteins, include various defensive proteins, transport proteins and enzymes etc. whereas, regulatory proteins include transcription factors, protein kinases and proteinases. The cis- and trans- acting elements that function in stress responsive gene expression have been exactly analysed to explain the molecular mechanisms of gene expression in response to various stress factors. Quantitative Real Time-

Polymerase Chain Reaction (qRT-PCR) is reported as one of the identical useful techniques for gene expression studies in plants (Higuchi *et al.* 1993) [13]. It is one of the most unfailing methods for gene expression study.

## Materials and methods

### 1. Seed Collection and Stress Treatment

The seed material of Kodo millet accessions (IPS-145, IPS-351 and IC-426676) for the present study was procured from International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) and National Bureau of Plant Genetic Resources (NBPGR), Hyderabad, India. Experiment was conducted in Herbal Garden of Acharya Nagarjuna University. Forest bags were filled with red soil and vermi compost in a ratio of 4:1. Five seeds per pot were sown and the distance between plant to plant was maintained as 10 cm. Few pots were subjected to NaCl stress treatments i.e 200mM,400mM and untreated plants were maintained as controls. After both treated and untreated leaves were collected immediately flash-frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  for subsequent use.

### 2. RNA Isolation and Reverse Transcription

Total RNA was extracted from leaf samples of respective treatments according to the protocol described by (Datta *et al.* 1989). Further, RNA was precipitated selectively using

lithium chloride ( $\text{LiCl}_2$ ). All RNA samples were further treated with RNase-free DNase (Fermentas), according to the manufacturer's instructions. RNA quantity and quality was assessed spectrophotometrically and the samples showing A260/A280 ratio of 1.8-2.0 and an A260/A230 ratio of 2.0-2.2 were used for subsequent analysis. Total RNA (20  $\mu\text{g}$ ) was separated following formaldehyde denaturing gel electrophoresis (Sambrook *et al.*, 1989) [28]. First strand cDNA was synthesized by reverse transcribing 2  $\mu\text{g}$  of total RNA with Revert Aid. First Strand cDNA Synthesis Kit (Fermentas) in a 20  $\mu\text{l}$  reaction using oligo (dT)<sub>18</sub> primers and RevertAid™ M-MuLV Reverse Transcriptase according to manufacturer's instructions. Reverse transcription was performed at  $42^{\circ}\text{C}$  for 1 hour followed by  $70^{\circ}\text{C}$  for 5 min. The cDNA was diluted 8 times for the use of real-time qRT-PCR reaction. All cDNA were stored at  $-20^{\circ}\text{C}$  until use.

### 3. Primer Design

Twenty-one gene sequences that are described in the literature from other millets were selected. Sequences were searched BLASTN algorithm was used to find the best hit against sequences from other millets. Twenty-one primer pairs were designed from these sequences (110 bp max length of the amplicon, optimal Tm at  $55^{\circ}\text{C}$ , GC% between 30% and 80%) with Primer 3.0 software. Primer sequences and the names of the genes are given in (Table 1)

**Table 1:** List of Primer Sequences used for qRT-PCR amplification of 21 different Genes

S.No	Name of the Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
1	Zinc finger (ZF)	TCGTGGCGGACCTCATG	CACGTCGTCATCCATCACTGA
2	RNA recognition motif (RRM)	CAGCAGCAACAGTGAGATCCA	CACCAATCACAGCACCTGAAA
3	NAC Transcription factor (NAC)	TGGTGAGGCTTGTGGAGTCA	CCCCAAGAGGGAAGAGATCAT
4	Auxin response factor 2 (ARF2)	TCCCATCCGTCTGGGTTTAG	GCAAACCGCGGAGAAGTAAAT
5	C3HC4-type RING finger (C3HC4-RF)	CACACCTGAGCCAAGCGAAT	GGCCCTGTCTGGTTCATCT
6	GAMYB-binding protein (GAMyb)	ACACGACGGCTATGGATGTG	TGTTTCATCGTCTCCCAAGAGAT C
7	Mitogen-activated protein kinase 12 (MAPK12)	TGTCGATGGATTGTCTGAAAAAGT	TGCCCGGGTCTTTGGA
8	Protein phosphatase 2C (PP2C)	GCTTGTGGTTGCGAATGTTG	CCGCCACACCATTTTTGC
9	Protein kinase domain protein (PK)	GAGCTGCTTTGGACGAGGAT	TCGATTGACTCTGCTGAAACTCA
10	CBL-interacting protein kinase 30 (CIPK30)	GCTGCTCCAACACTTTCTGTGA	GCAGCGGTTTTGCATCTTC
11	Calcium dependent protein kinase (CDPK)	CAGAATTGACAGAGAATGAAATCCA	GATGGTCCGCTGTGTCAATA
12	ATP-dependent Clp protease (ATP-CP)	AGTTTCGCCGCACCACTAGT	CACGGAGGGCTCCTCATG
13	Subtilisin-chymotrypsin inhibitor 2 (SCI)	CGCTCCGCCTTGATCTTC	TGAGCTAAAGGGATGCACGAT
14	MRP-like ABC transporter (MRP-ABCT)	GCGCAAATCAACCCCTCATA	CCGATAGTTGGGTCCACGAT
15	UVB-resistance protein (UVR8)	TTTGGGTTTGGCGACAATG	CAAGCACACACGAATGTATTTCATG
16	Senescence-associated protein (SAP)	TCGATGTCGGTCTTCCTATCA	TGGGTGAACAATCCAACACTTG
17	UDP-glucose-4-epimerase (UGE)	AGCAGCACCGACCAACTTTT	TTGCAGGAGACGCTACGATTT
18	Nucleosome assembly factor	GCTGCACTCCGAGGAAGT	CACGGAGAGCTTTCAACGGATT
19	NAC2 protein	CCTTCCGCAGCCTTGGA	ACATGTTGCTGAAATGGCTGTT
20	Glutathione reductase	TGGACGTCGGGCTCACA	TCGGCTGCTGAAGAGTCTGTAA
21	Actin	GCAGCGGGTCATGGATTC	AGCGAGCAGCGGTAGACAA

### 4. Quantitative RT-PCR

The expression study was performed using a 96 well plate on Light Cycler 480 (Roche) with LightCycler®480 SYBR Green I Master (Roche). The reactions were performed according to the manufacturer's instructions. The PCR program was initiated at  $95^{\circ}\text{C}$  for 5 min to activate *Taq* DNA polymerase, followed by 35 thermal cycles of 10 seconds at  $95^{\circ}\text{C}$ , 10 seconds at  $55^{\circ}\text{C}$  and 15 seconds at  $72^{\circ}\text{C}$ . Melting curve analysis was performed immediately after

the real-time PCR. The temperature range used for the melting curve generation was from  $55^{\circ}\text{C}$  to  $95^{\circ}\text{C}$ . All assays were performed using three technical and biological replicates, a non-template control and a non-RT control.

### 5. Primers Specificity and Amplification Efficiency

The determination of the crossing amplification point (Cp) as well as the relative quantification analysis ( $\Delta\Delta\text{CT}$ -

method) was performed using the Light cycler 480 software 1.5. The amplification of non-template controls generated Cp values above 45 was not detectable.

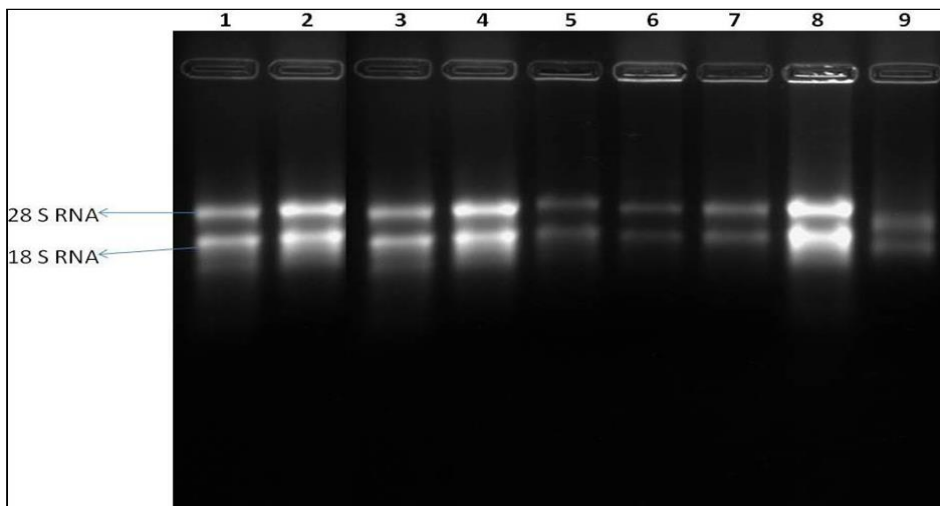
**6. Software and data analysis**

The average Cq values calculated from RT-qPCR data for all samples or genes were used for further stable gene identification. The data were analyzed by geNorm v3.5 (Vandesompele *et al.* 2002) [32] and Norm Finder (Andersen *et al.* 2004) [4] version 2 whereas the raw Cp values were imported into Best Keeper version 1 (Pfaffl M *et al.* 2004) [23, 24] were applied to compare the expression stability of the reference genes across all treatments.

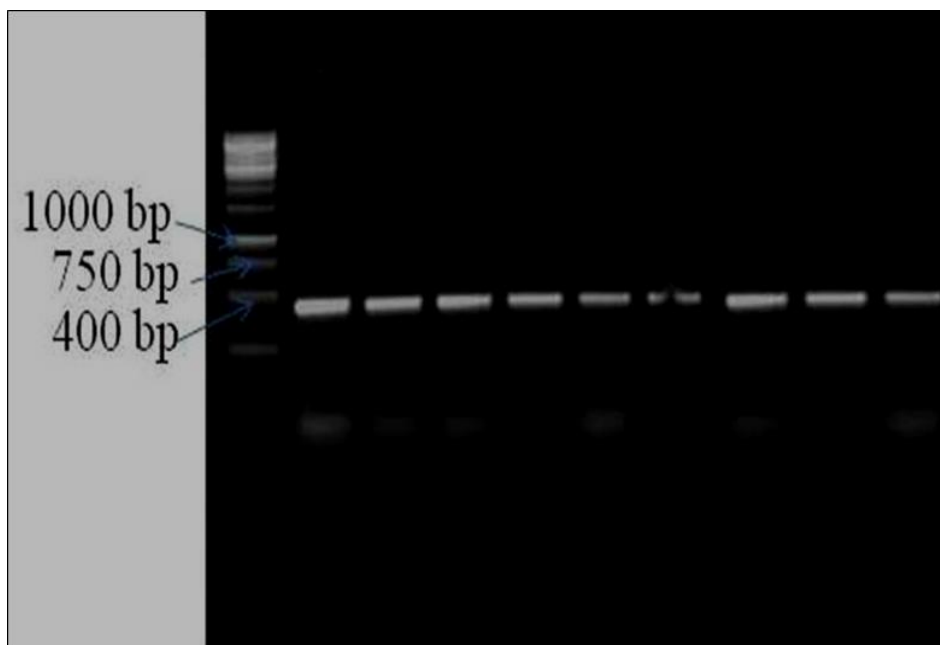
**Results and discussion**

**1. Quality and Integrity of Extracted RNA**

The purity and quantity of RNA extracted from controls, 200 mM and 400 mM samples was analysed at A260/280. All the genotypes were varied significantly in production of RNA both varietal wise and treatment wise (Table 2). Further RNA isolated from all the genotypes was reverse transcribed and the size range of the synthesized cDNA showed intact of mRNA and minimal degradation (Figure 1). PCR amplification of cDNA obtained from extracted RNA was consistent, the target bands were visible and distinct, and were the expected size of the actin gene fragment targeted by the primers used (Figure 2). All genotypes have produced the required amounts of RNA, suggesting that it can be further useful in RT-PCR studies.



**Fig 1:** The intact mRNA in Control and treated leaf samples of Kodomillet



**Fig 2:** Actin amplification products of cDNA prepared from control and stressed leaves

**Table 2:** ANOVA of Quantity and Purity of extracted Total RNA from leaves of kodomillet

Variety	IC 76 Tolerant			IPS 351 Moderately tolerant			IPS 145 Susceptible		
Treatment	Control	200 mM	400 mM	Control	200 mM	400 mM	Control	200 mM	400 mM
OD at 260/280 nm	0.07/0.04	0.08/0.05	0.09/0.05	0.09/0.05	0.07/0.04	0.07/0.04	0.07/0.04	0.07/0.05	0.07/0.04
Quantity (µg/µl) 260 × 40/1	2.8	3.2	3.4	2.8	2.8	3.4	2.8	2.8	2.8
Purity at 260/280	1.60	1.73	1.82	1.70	1.79	1.73	1.65	1.70	1.71

## 2. RT-PCR Data Analysis

An effective stress perception and a rapid integration process are both important components of early acclimatization to salinity stress. The up-regulation signal transduction genes (mitogen activated protein kinase12 (MAPK12) (Figure 3), protein phosphatase2C (PP2C), protein kinase, calcium dependent protein kinase (CDPK) (Figure 4), CBL-interacting protein kinase30 (CIPK30) and which were induced at 200mM of salt concentration in cv. IC-426676, although their abundance decreased at high salt concentration. MAPK genes have been associated with signal transduction pathways induced by salinity, dehydration and osmotic stress (Moustafa *et al.* 2008 [21] and Puranik *et al.* 2012) [25]. PP2C is differentially expressed in both *Arabidopsis thaliana* and barley in response to various abiotic stresses (Seki *et al.* 2002 [29] and Talame *et al.* 2007). The early accumulation of CDPK and CIPK transcripts in IC 426676 agrees well with the reported involvement of Ca<sup>2+</sup> signal transduction in stress response (Kolukisaoglu *et al.* 2004). The specific activation of these signalling transcripts in the tolerant cultivar exposed to salinity stress suggests that it has more effective signal perception machinery than does IPS 351 and IPS 145.

The control exerted by transcription factors over downstream stress-responsive gene expression is a second crucial component of the plant stress response. Most of the transcription factor genes i.e zinc finger (ZF), RNA recognition motif (RRM), NAM/AFAF1,2/CUC2 (NAC and NAC2), auxin response factor2 (ARF2), C3HC4-ring finger (C3HC4-RF) and gibberellin associated MYB-binding protein (GAMyb) were up regulated at low salt concentrations (200 mM). However, their expression levels were decreased at high salt concentrations (Figure 5, 6 and 7). Although most of these transcription factors have been implicated in stress-inducible gene expression (Hu *et al.* 2006 [14] and Ma *et al.* 2012), the functional implication of ARF and GAMyb in salinity stress has not yet been fully established (Baisakh *et al.* 2008 [5] and Jain and Khurana 2009) [15]. Their relatively higher level of expression observed in the tolerant cultivar IC 426676, however suggests a role in adaptation to low salinity stress. Low expression levels in the IPS 145 correlated with our physiological data.

Plant survival in a saline environment involves the sequestration of Na<sup>+</sup>, which is generally mediated by transporters, symporters and antiporters. The expression of a Na<sup>+</sup> dependent transporter-like, inorganic H<sup>+</sup> pyrophosphatase, an ABC transporter, a mitochondrial carrier-like protein and a putative sugar carrier protein C were all preferentially up-regulated in salinity stressed IC-

426676. The expression of an MRP-like ABC transporter increased gradually with increased salt concentration in cv.IC-426676, but its level was considerably lower in cv. IPS-145 than in IC-426676. Rodrigues *et al.* (2009) [27] have also reported that an MRP-ABCT is expressed at similar levels in both drought tolerant and sensitive sugarcane plants. Its activation is associated with the elimination of potentially toxic compounds from the cytosol. The preferential expression of Na<sup>+</sup> dependent transporter like and inorganic H<sup>+</sup> pyrophosphatase genes in tolerant cultivar might likely function in Na<sup>+</sup> exclusion from the cytosol through proton dependent way, as proposed in Arabidopsis (Yang *et al.* 2009 [34] and Silva and Geros 2009) [30].

The lower Na<sup>+</sup> and higher K<sup>+</sup> and Ca<sup>2+</sup> levels accumulated by IC 426676 during salinity stress would be expected to reduce ion toxicity by stabilizing the cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio. The cell responds to stress by synthesizing various chaperones and proteases, as these are important for the refolding or degrading of stress-damaged proteins. In the present study proteases that were differentially expressed involved in selective. Two protease genes ATP-dependent Clp protease and subtilisin-chymotrypsin inhibitor (SCI) involved in protein degradation were more strongly expressed in cv.IC-426676 than in IPS-145. Clp proteolytic complexes are ATP-dependent proteases which act in an energy dependent manner. Protease inhibitors such as SCI have been reported to be induced by various stresses (Richards *et al.* 1998) [26].

The expression of UDP-glucose-4-epimerase (UGE), endo chitinase and a xylanase inhibitor gene was found high at 200mM salt concentration, but its expression level was low at high salt concentration. Similarly, drought stress induced the expression of a UGE gene in rice (Nguyen *et al.* 2004). The abundance of two stress and defense-associated transcripts UVB-resistance protein (UVR8) and a senescence-associated protein (SAP) in response to salinity treatment as evaluated by qRT-PCR showed that these genes were also differentially expressed between the three cultivars. The UVR8 gene was induced in IC-426676 by about 6-fold 200mM salt concentration and its expression was increased with salt concentration, but its expression levels in the susceptible cv. IPS-145 were very less. UVR8 has been reported to be involved in protecting gene expression under adverse conditions (Brown *et al.* 2005) [6]. The SAP transcript levels were very at higher concentration of salt in all the genotypes, however its expression was very high in the IPS-145. This gene is more strongly induced in heat sensitive than in heat-tolerant plants (Zhang *et al.* 2005a) [35].

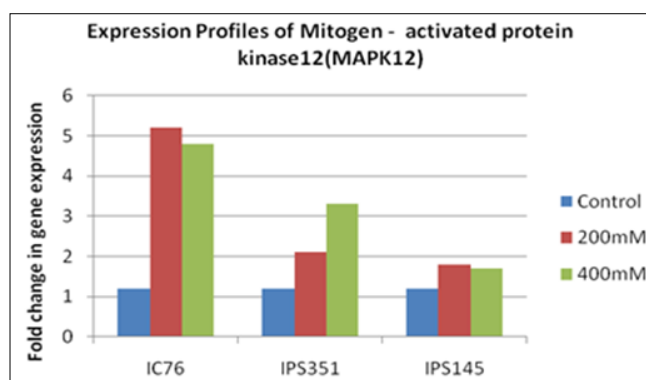


Fig 3: MAPK 12 expression profile

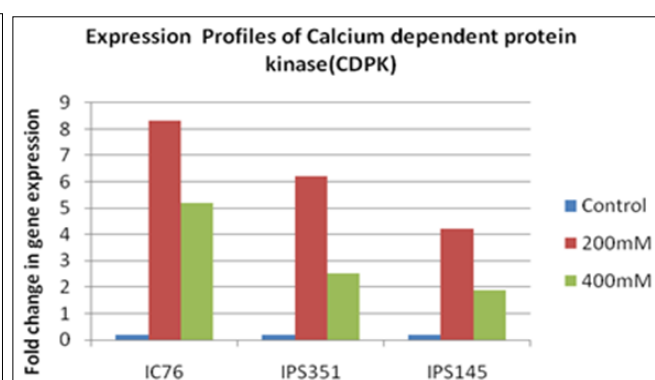


Fig 4: CDPK gene expression

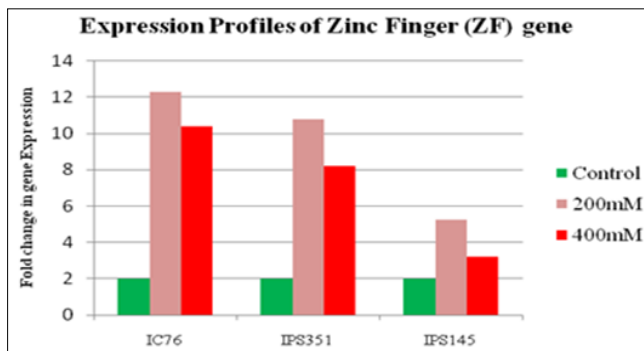


Fig 5: Zinc Finger gene expression profile

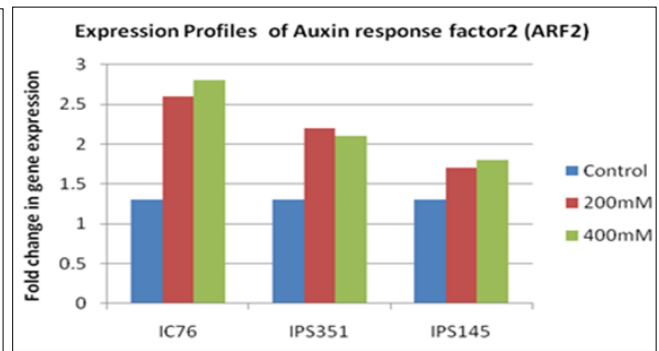


Fig 6: ARF2 gene expression profile

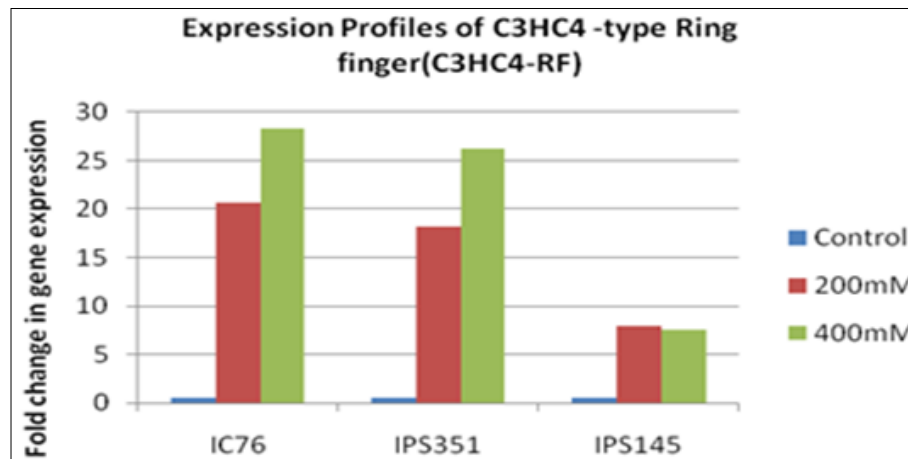


Fig 7: Ring finger (C3HC4-RF) expression

## Conclusion

Based on the mean performance of three kodo millet germplasm under NaCl stress conditions, expression of salt stress responsive genes, the accession IC 426676 was proved as the best salt tolerant germplasm. Hence this accession can be recommended as suitable to grow under salt stress conditions. Further, IC 426676 germplasm has a scope to exploit them in future plant breeding and transgenic experiments to improve salt tolerant kodo millet germplasm.

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## Declaration of interest statement

The authors declare no conflict of interest.

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