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Transcript analysis of differentially expressed genes in minor millets under water stress

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Abstract

Drought stress is one of the abiotic stresses which may alter plant growth, metabolism and yield. Millets are resilient to extreme environmental conditions especially to inadequate moisture and are rich in nutrients. The aim of this research was to analyze the expression of some key genes in minor millets under drought stress by using semi-quantitative RT-PCR. Expression profiling of seven drought responsive genes revealed that the transcripts showed a differential expression pattern in minor millets under water stress treatment when compared to their control counterparts. Expression of genes was up regulated by water stress treatment, suggesting that they may involve in the drought tolerance response of the crop. Further efforts in validation of identified genes can be used as candidate genes for development of water stress tolerant transgenic in other related crops.

Keywords: Drought, semi-quantitative RT-PCR, Abiotic stress

1. Introduction

Abiotic stresses like drought, temperature, salinity and mechanical injury on plants found to create a negative impact on crop productivity^[1]. Water stress may occur at any time during the growing season because of variable climatic changes associated with global warming and this may lead to a profound decrease in yield^[2]. Breeding drought tolerant crops is one way to increase grain yield, but, progress has been slow during the past decades due to lack of understanding of the traits and mechanisms of drought tolerance^[3]. It is important to identify the critical period and responses to water deficit among crops. Crop plants will face a greater range and number of environmental stresses. So it is very urgent to breed stress-tolerant crop varieties to satisfy an increasing demand for food productivity due to global population increase^[4, 5].

Minor millets also called small millet are a group of grassy plants with short slender culm and small grains. They are categorized as coarse cereals and mainly form staple food for the tribal people where cultivation of major cereals like rice, wheat and maize is either not popular or fail to produce substantial yield^[6]. Millets are examples of less-utilized crops with adaptation to marginal lands where they can withstand various stress conditions and contribute to sustainable low-input food production. Local farmers value the small millets for their nutritional and health, tolerance to extreme stress including drought, and ability to grow under low nutrient input conditions, ideal in an era of climate change and steadily depleting natural resources. Little scientific attention has been paid to these crops; hence they have been termed "orphan cereals." Despite this challenge, an advantageous quality of the small millets is that they continue to be grown in remote regions of the world which has preserved their biodiversity, providing breeders with unique alleles for crop improvement^[7].

In India, small millets are cultivated in the semi-arid and hilly regions inhabited by traditional farmers. Finger millet is the principal crop amidst the small millets, occupying 60–70% of the total area under small millet production globally, followed by kodo millet, foxtail millet, little millet, proso millet, and barnyard millet. Grains of small millets are extremely resistant to storage pests and can be stored for indefinite periods^[8]. Nutrition wise, grains of small millets are rich in micronutrients, particularly calcium and iron. They have high dietary fiber content, rich essential amino acids, and low glycemic index^[9, 10]. Minor millet is known for its great level of tolerance against drought, salinity and diseases. It is necessary to dissect the transcriptome information under different stress conditions to prospect novel genes.

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Characterization of drought responsive genes in these millets will provide valuable information on molecular mechanism and dynamics underlying their drought tolerance potential. Important genes identified through such investigation can serve as useful/ novel candidates for improvement of drought tolerance characteristic in other crops as well. With these considerations, the current study was undertaken to characterize drought tolerance mechanism in millets at biochemical and molecular level.

2. Material and methods

2.1 Plant Material

The experimental materials of the present investigation comprised of minor millet were planted in pots separately and maintained in green house at 28 ± 2 °C. Pots were watered normally (once per day) until the plants attain 21day period (Figure 1). The 21-day-old seedlings were subjected to water stress. Water was withdrawn from pot on 21th up to 29th days. Sample were harvested on 30th days from both control and water stressed plants in liquid nitrogen and stored at -80 °C for RNA isolation.

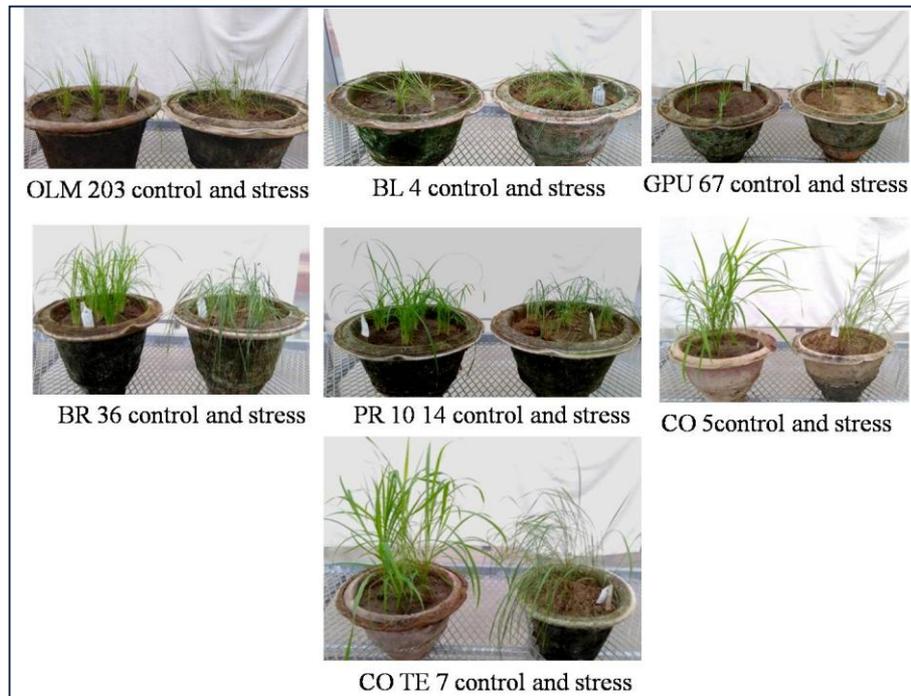


Fig 1: Minor millets genotype under control and water stress condition

2.2 Total RNA Extraction

Total RNA was isolated using TRIzol (Invitrogen, USA) using a manufacturer's protocol with minor modifications and the concentration was determined using Nanodrop spectrophotometer ND-1000® (NanoDrop Technologies, USA). cDNA was synthesized using BIORAD iScript™ cDNA Synthesis Kit as per manufacturer's instructions.

2.3 Semi-Quantitative RT-PCR

Semi-quantitative RT-PCR reactions were carried out with 20 µl of the reaction solutions using gene specific primers and Actin gene primers as internal controls. The reaction was

performed by adding following components in order to sterile thin-walled PCR tubes for each PCR amplification reaction: 11µl of RNase-free water, 2µl of 10X PCR Buffer, 2µl of dNTP mix (2mM each dNTP), 1µl of primer, 2µl of experimental first-strand cDNA reaction, 0.5µl of *Taq polymerase* (5U/ul). The annealing temperature for each primer and amplified fragment size is given in Table 1. Amplifications were performed by a cycles of: 2 min at 95 °C followed by 35 cycles each of 15 sec at 95 °C, 30 sec at 56 - 60 °C, and 30 sec at 68 °C, and final extension of 1 min at 68 °C .

Table 1: Details of primer used for semi quantitative RT PCR analysis ^[11].

S.N	Primer ID	GenBank Acc. No.	Forward and Reverse sequence (5'-3')	Amplicon length (bp)
1	NAC2	GT090919	CCTTCCGCAGCCTTGGA ACATGTTGCTGAAATGGCTGTT	56
2	CDPK	GT090918	CAGAATTGACAGAGAATGAAATCCA GATGGTTCCGCTGTTGTCAATA	68
3	U2-snRNP	GT090867	TGTGACCGACTTCCGTGAAG CCACGGTTGCAGCTGTCT	60
4	Synaptotagmin	GT090932	TCTTGCAAGGTGCCAAATCTG GGCTGTGGCGTCCACTTAA	58
5	Aquaporin	GT090849	CCCGTTCAAGAGCAGGTCTTA CCTGTTTGGACTGGCATCTCA	71
6	MPK 17-1	GT090884	TGTCGATGGATTGTCTGAAAAAGT TGCCGGTCTTTGGA	61
7	Scythe protein	GT090877	CCAGACACTAGCAGCACACATG CATCCCTTGCTCTGTTTGA	61

2.4 Gel Electrophoretic Analysis and Gel Elution

Separation of amplified fragments was carried out using Bio-rad gel electrophoresis assembly. PCR amplification products were analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide solution (0.5 µg/ml). The gel was run in 1X TBE buffer at 70-80 Volts for 45 minutes to 1.5 h. Standard ladders of 100bp and 1kb from (Fermentas

TM) sizes was used. The resultant PCR product was then resolved on 1.5% Agarose gel followed by digitalization of fluorescence data to numerical values using GelQuant.NET Analyze. The relative expression of genes was expressed in terms of fold change under water stress with respect to their control (Figure 2).

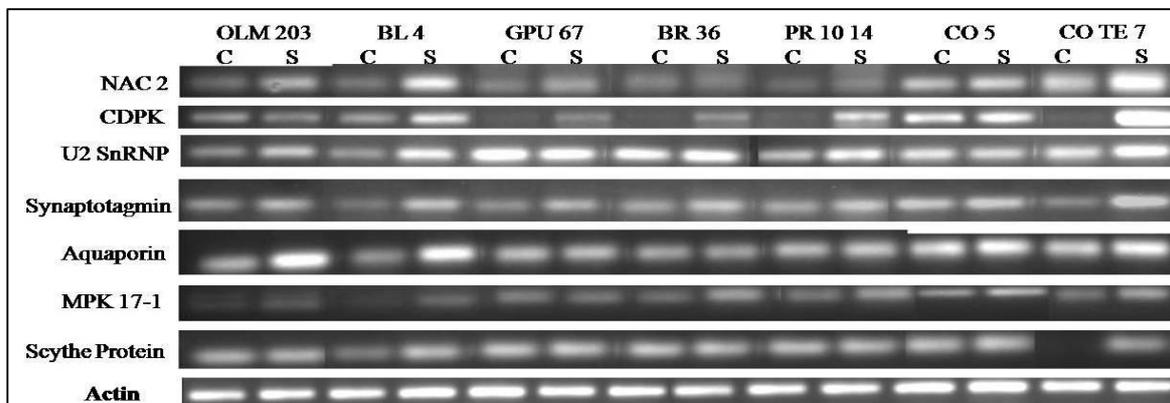


Fig 2: Semi quantitative RT PCR analysis of minor millet genotype under control and water stress condition

3. Result and discussion

3.1 Expression pattern of dehydration stress responsive gene in minor millet genotypes under water stress

Semi quantitative RT-PCR was performed to analyze the expression pattern of seven differentially expressed up regulated transcripts in minor millet under water stress and control condition. The genes include NAC2, Calcium-

dependent protein kinase, U2-snRNP, Plant Synaptotagmin, Aquaporin, MPK17-1, and Scythe protein induced by water stress. Semi quantitative RT-PCR analysis showed differential expression of these seven transcripts in millet genotypes under stress with respect to the control condition. The results are discussed below in detail.

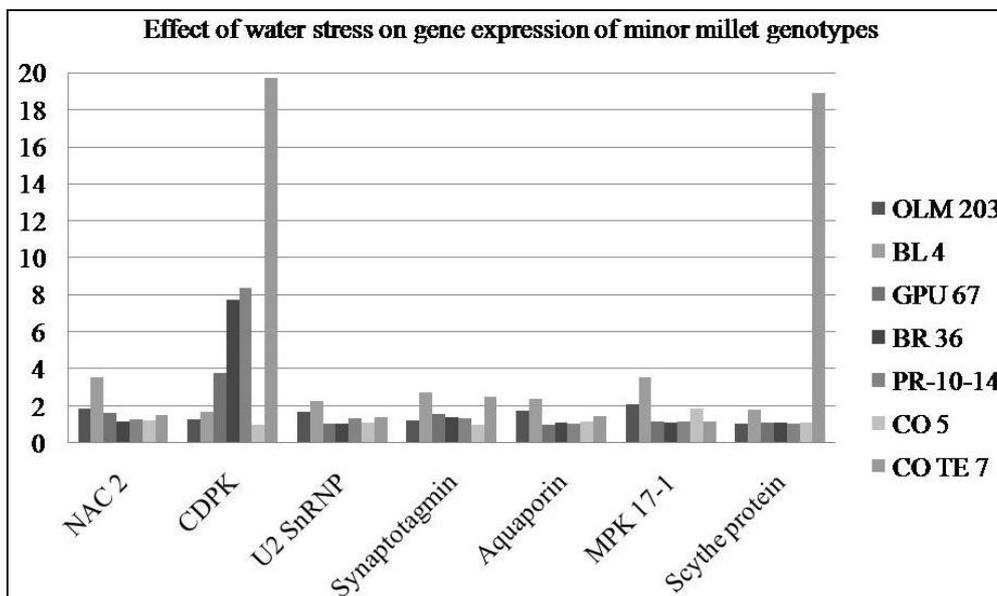


Fig 3: Effect of water stress on gene expression of minor millet genotypes (Fold increase)

NAC (NAM, ATAF, and CUC) is a plant-specific gene family of transcription factors. The Semi quantitative RT-PCR analysis of NAC2 protein-like transcript accumulation increased with water stress up to 3.5-fold in little millet genotype BL 4 followed by OLM 203 (1.8 fold up-regulation) when compared to drought tolerant genotype CO TE 7 (1.5 fold) (Figure 3). Stress responsive NAC TFs may have important roles in providing tolerance against abiotic stresses and their over-expression can improve stress tolerance in crop plants. Transgenic rice plants engineered with a NAC TF (OsNAC6) were found to exhibit enhanced tolerance against various abiotic (drought and salinity) and biotic stresses in

rice [12]. Isolation and validation of the function of a novel NAC transcription factor namely EcNAC67 exhibiting contrasting salinity responsive expression pattern between the susceptible and tolerant finger millet genotypes [13]. Similarly, the expression profiles of 50 foxtail millet (*S. italic* L.) NAC genes analyzed in response to various abiotic stress, and found that *SiNAC110* was up-regulated following both dehydration and salinity stress; its expression level increased more than 9-fold in stress-treated plants [14]. The up regulation of NAC transcript under water stress suggests that it may play an important role in the cross-linking of different signaling pathways in minor millet.

Calcium-dependent protein kinases play important roles in signaling pathways for various stress responses [15, 16]. Semi quantitative RT-PCR of CDPK showed up-regulation under water stress up to 19.7 fold in previously reported drought tolerant CO TE 7 genotype followed by 8.7 fold in finger millet genotype PR 10 14. This induction was found to be 7.7 fold in BR 36 (Figure 3). In several previous studies, induction and expression of CDPK(s) have been reported to be higher in tolerant cultivars under different abiotic stresses [17, 18]. Drought stress at whole plant level significantly induced the expression of *EcCIPK31-like*, indicating that the gene is linked to drought signaling pathways [19]. Over expression of GhCIPK6 has been reported to significantly enhances the tolerance to salt, drought and ABA stresses in transgenic *Arabidopsis*, indicating that GhCIPK6 acts as a positive regulator in response to salt and drought stress, and is supposed to be a potential candidate gene to improve stress tolerance by genetic manipulation in cotton and other crops [20]. The CIPK family of 26 protein kinases regulates the function of several ion transporters at the cell membrane to restore ion homeostasis under stress situations [21]. The differentially induced expression of OsCIPK genes by different stresses and the examples of improved stress tolerance of the OsCIPK transgenic rice suggest that rice CIPK genes have diverse roles in different stress responses and some of them may possess potential usefulness in stress-tolerance improvement of rice [22].

Alternative splicing takes place in highly specialized structures within nucleus called spliceosomes consisting of five small nuclear ribonucleoprotein particles, snRNPs (U1, U2, U4/6, and U5) and other non-snRNPs [23]. In this study the expression of U2-snRNP was 1.6 and 2.2 fold higher than control in little millet genotype BL 4 and OLM 203 respectively (Figure 3). Similar gene induction of U2-snRNP has been reported among tolerant and susceptible cultivars of Foxtail millet [11]. This induction was found to be temporally regulated. Their findings suggest that U2-snRNP may play a significant role in alternative splicing in foxtail millet and thus regulating gene expression.

In this study the expression of Synaptotagmins was found to be 2.7 fold higher than control in little millet genotype BL 4 followed by 2.4 fold in CO TE 7 and 1.5 fold in finger millet genotype GPU 67 under water stress condition with respect to corresponding control plants (Figure 3). Tolerant cv. Prasad showed around 2.5-fold inductions at early stages of dehydration stress, while in cv. Lepakshi the mRNA accumulation was comparable to control at all-time points. Up-regulation of Synaptotagmin in dehydration stress indicates its role in stress signal transduction and tolerance [11]. It has been shown that Synaptotagmin 1 protein (SYT1) imparts calcium dependent freezing tolerance via membrane resealing, and also loss of function of this gene reduces cell viability and plasma membrane integrity in *Arabidopsis* [24, 25]. Aquaporin belongs to major intrinsic protein super family which functions as a membrane channel. In this study semi quantitative RT-PCR analysis showed the expression of Aquaporin was 2.4 fold higher than control in little millet genotype BL 4 followed by 1.7 fold in OLM 203 and 1.4 fold in CO TE 7 under water stress condition (Figure 3). Level of water channel protein RWC3 mRNA was increased in upland rice after 4 h of the PEG treatment, whereas there were no significant expression changes in lowland rice [26]. Over-expression of a Panax ginseng tonoplast aquaporin enhances drought and salt tolerance ability in transgenic *Arabidopsis* plants [27].

MAP kinase signaling is one of the most important and conserved pathways in most cellular process as well as environmental stress responses [28, 29]. The results showed that the expression of MAP kinase was 3.5 fold higher than control in little millet genotype BL 4 followed by 2 fold in OLM 203 and 1.8 fold in CO 5 under water stress condition (Figure 3). MAP kinase gene has been reported to be induced due to dehydration, salinity and hyper-osmotic stresses [28].

Scythe protein has been observed as an apoptotic regulator in vertebrates and is an essential component in reaper induced apoptosis [30, 31]. Semi quantitative RT-PCR analysis showed the expression of Scythe protein was 18.8 fold higher than control in CO TE 7 followed by 1.8 fold in little millet genotype BL 4 under water stress condition (Figure 3). Scythe protein has been observed to be higher in the tolerant cultivar Prasad at all-time points of dehydration stress in comparison to a drought sensitive cv. Lepakshi [11].

4. Conclusion

Present study helps us to identify the transcripts expressed in response to water stress in minor millets. To the best of our knowledge, this is the first report about analysis of differentially expressed transcripts in minor millet under water stress that includes genes reported previously for dehydration stress in foxtail millet. The induction of these genes under water stress suggests their function in possible regulation of water stress adaptation in minor millets. Induction of NAC2, Calcium- dependent protein kinase, U2-snRNP, Plant Synaptotagmin, Aquaporin, MPK17-1, and Scythe protein transcript suggests that this gene might impart drought avoidance capacity to the tolerant cultivar. Further efforts in validation of identified genes can be used as candidate genes for development of water stress tolerant transgenic in other related crops.

5. Acknowledgments

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