Comparative study of calcium binding protein expression in developing spike of two different contrasting genotypes of finger millet (Eleusine coracana L.)

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Abstract- Calreticulins are a high-capacity calcium binding proteins (CaBPs) comprising the major intracellular storage reservoir. Finger millet is rich in calcium; it accumulates upto 450 mg Ca/100g of seed. *Ec_CRT1* is identified as one of the calreticulin present in the transcriptome of the fingermillet developing spike. But there is little molecular information present about it in different genotypes of finger millet. Such information when available would be immensely helpful in the genetic engineering of common cereals for high seed calcium contents and in turn be an important step forward to alleviate the problem of calcium deficiency. In order to understand the mechanism of high calcium accumulation by *Ec_CRT1*, two genotypes of finger millet differing in seed calcium content i.e. GP-1 (low calcium) and GP-45 (high calcium) were selected in present study. The differential expression was analyzed in developing spike of both genotypes and it was observed it is highly expressed in GP-45 as compared to GP-1 in later stages. This high expression of genes in later stages could be utilized in future biofortification programme of the Finger millet.

Key Words- Developmental, Expression, Finger millet, Genes, Proteins

1 INTRODUCTION

Nutrients ensures normal metabolism, physical well being and growth [1]. Minerals are one of the micronutrients which are inorganic in nature and play a key role in ensuring good health and well being. Approximately 4% of the body's mass consists of minerals. They can be classified into trace and macro elements. Macrominerals constitute a larger percent of the body and are needed in more amounts, as compared to micro minerals. They are found in small quantities within the body in ionized form and can be obtained from a wide variety of food. Finger millet (*Eleusine coracana*) is rich in mineral contents and proteins as compared to other cereals [2]. One of the most attractive features of finger millet grain, it contains exceptionally high level of calcium which is much higher compared, to other cereals and millets. It accumulates upto 450 mg Ca/100g of seed.

Calcium is an essential macromineral for growth and development of plants. It plays both metabolic as well as structural role in plants. Calcium signaling mechanisms are widely employed by all eukaryotic organisms to regulate gene expression and a variety of other cellular processes [3,4]. Plant cells are equipped with highly efficient mechanisms to perceive, transduce and respond to a wide variety of internal and external signals during their growth and development. Perception of signals via receptors results in generation or synthesis of non-proteineous molecules often termed messengers such as calcium which control diverse cellular processes through calcium sensors which is also known as calcium binding proteins [5]. Apart from this transmission of calcium signal, the CBPs accumulates the calcium which is responsible for a high calcium accumulation in the different tissues. In the plant kingdom, calcium binding proteins regulating Ca2+ homeostasis exhibit diverse features, including calcium binding affinity, molecular mass, and gene expression.

The calcium-binding proteins (CBPs) which can be categorised into EF-Hand or Non EF-Hand proteins. EF-hand proteins are involved in a wide variety of physiological processes, including signalling, cell cycle regulation, second messenger production, muscle contraction, and vision. An enormous and diverse range of target proteins that belong to different protein classes like transporters, cell structure proteins, metabolic enzymes, signalling proteins and transcription factors are regulated by calcium binding proteins (6). Calreticulin (CRT) is one of the most abundant Ca2+ binding proteins resident in the ER, with an established role as a molecular chaperone [7] and it is highly stable, with a relatively long lifetime (half-time about 26 h). It increases the Ca2+ storage capacity of the ER and modulates the function of ER Ca2+ ATPase. It is responsible for the main Ca2+-retaining pool in plants. Ca2+ enters the ER through sarcoplasmic reticulum and ER Ca2+-activated ATPase (SERCA) pumps and is bound to proteins such as CRT and grp78 (BiP) [8]. It is a high-capacity CaBP comprising the major intracellular storage reservoir (8) and has a single high-affinity and multiple low-affinity calcium binding sites. It has previously been known by several other names (e.g. high-affinity CaBP, calregulin, ERp60, CRP55, CAB-63, CaBP3, calsequestrin-like protein). *Ec_CRT1* gene belongs to calreticulin superfamily of calcium binding proteins. In earlier study an amplicon of 600bp was amplified using the conserved primers from different cereals and the expression was studied in GP45 genotype [10].

Here in this study, in order to understand the mechanism of high calcium accumulation, full length ORFs was identified from the transcriptome available and two different contrasting genotypes differing in grain calcium content of finger millet were selected for expression study . An attempt has been made to have a comparative study of *Ec_CRT1* gene for understanding their immense role in

differential accumulation of calcium in finger millet genotypes.

2 MATERIAL AND METHOD

2.1 Collection of Developmental stages of Spikes in Fingermillet.

Different stages of developing spikes are collected from high calcium accumulating genotype (GP- 45: 452.8/100g seed) and low calcium accumulating genotypes (GP-1: 117.56 mg /100g seed) of finger millet. The stages are collected as S1 (spike emergence); S2 (pollination); S3 (dough) and S4 (maturation). These four stages are collected on the basis of developmental stage of ovary and anther.

2.2 Isolation of total RNA from developing spikes of finger millet and cDNA synthesis

Total RNA was isolated from the different stages (S1 to S4) of developing spikes using the total RNA isolation iRIS system (developed by IHBT, Palampur). RNA preparations were subjected to DNaseI treatment according to manufacturer's instructions (Fermentas) and integrity of total RNA was analyzed through 1.2% agarose gel electrophoresis. Two micrograms of total RNA of each sample was used to synthesize first-strand cDNA by oligo (dT)18 primer using Revert Aid™ H-Minus First strand cDNA synthesis kit (Fermentas).

2.3 Expression profiling of *EcCRT1*gene in different stages of developing spikes of two finger millet genotypes.

Total RNA was isolated and cDNA was synthesized as described above. Real-time PCR was carried out using the five Prime Real Master Mix SYBR ROX (Eppendorf India Limited, Chennai, India) according to manufacturer's instructions. The primer for Ec_CRT1 gene for performing real time were designed and given in Table 1 which will amplify amplicon of their respective size. Finger millet tubulin gene isolated in our lab was taken as internal control to normalize the expression level of the target gene. Ct values at different dilutions of cDNA were analyzed and compared for the reverse transcription efficiencies of different stages of developing spikes in finger millet and tubulin genes and it was found almost equal. The following amplification program was used: 95°C for 2 min, 40 cycles at 95°C for 30s, 58°C for 30s and 72°C for 30s while 1 cycle at 58°C for 15s and 95°C for 15s. All samples were amplified in triplicate and the relative expression of the gene in different stages of developing spikes was calculated by ΔΔCT method.

Name	Primer	bp	Amplicon size
Ec_CRT1F	AATTCGGCGGGGACACACCA	20	242
Ec_CRT1R	GGCATCAGGGCGGATGACCAA	21	

Table: 1 List of primer

3.0. RESULT AND DISCUSSION

3.1 Isolation of total RNA from developing spikes of finger millet and cDNA synthesis

To study the Transcript profiling of potential genes in different tissue, pure RNA with good yield is the first and foremost prerequisite of any expression study. In present study total RNA was extracted from spike sample of all stages (S1, S2, S3, S4) from both genotypes using total RNA isolation iRIS system from IHBT Palampur. RNA isolated is treated with DNase to remove DNA contamination which might interfere with the expression profiling of gene of interest by causing nonspecific amplification. Upon agarose gel electrophoresis of RNA isolated from all the specific tissues the presence of intact rRNA band along with smearing was revealed, which indicated good quality of RNA. After interpretation of quantification data it was found that all the samples have A260/A280 value approximately equal to 2, which implies pure and good quality RNA as shown in Fig:1. c-DNA was prepared from DNase free RNA of both genotypes (GP-1 and GP-45) for real time expression analysis. Prepared c-DNA was checked by using with tubulin specific primer and electrophoresed by using 1.5% gel with 100bp ladder as standard measurement. Specific band of tubulin gene was observed on the gel having size 180bp.

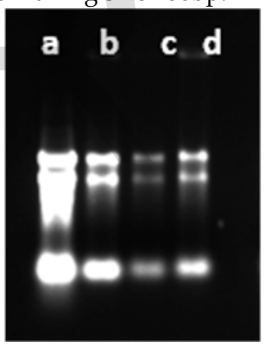

Fig: 1 RNA isolation from different stages of developing spike (S1-S4) in finger millet.

3.2 Expression profiling of Potential CBPs genes using Real time PCR in two different genotypes of finger millet.

Expression profiling study provides a means for characterization of tissue wide expression of specific gene and time dependent manner. Therefore expression profiling of potential candidate genes were performed in different stages of two genotypes of finger millet which are differing in their calcium content. And efforts were made to correlate the expression pattern of each potential gene with calcium accumulation and transport. By using c-DNA of GP-1 and GP-45 as template, a specific band (for tubulin) was confirmed. In order to determine the exact quantitative expression of the *Ec_CRT1* gene, a real time quantitative PCR analysis was performed. The expression of these genes was studied at 4 developing spike stages in both genotypes.

The tubulin gene was used as an internal standard to normalize any variation in the quantity and quality of the starting template c-DNA.

The *Ec_CRT1* genes was expressed in both genotypes but with differential expression pattern. The level of transcripts changes from S1 to S4 stages of developing spikes of both the finger millet genotypes. For *Ec_CRT1* gene, in GP-45, the transcript level was high in S1 stage, then it decreased in S2 stage and then it goes on increasing till S4 stage. At S4 stage the transcript level of *Ec_CRT1* was higher when compared to the other stages while in GP-1, the transcript level was at 1.0 in S1 stage, then it increases in S2 stage and then it goes on decreasing till S4 stage. At S4 stage the transcript level of *Ec_CRT1* was lower when compared to the other stages. The transcript level of *Ec_CRT1* was higher in GP-45 as compared to GP-1 at S4 stage (Fig: 2A). The results of real time expression analysis of *Ec_CRT1* also supported by the RNA-Seq FPKM expression values of LOC_Os07g14270.1 (homologus to the *Ec_CRT1*) taken from the rice genome annotation project database which are 220.059 and 499.465, 242.67, 83.4991, 9.25023 and 16.714 at post emergence, pre emergence influorescence, seed-5 DAP (days after pollination), Embryo- 25 DAP, Endosperm- 25 DAP and Seed- 10 DAP of nipponbare are respectively [10].

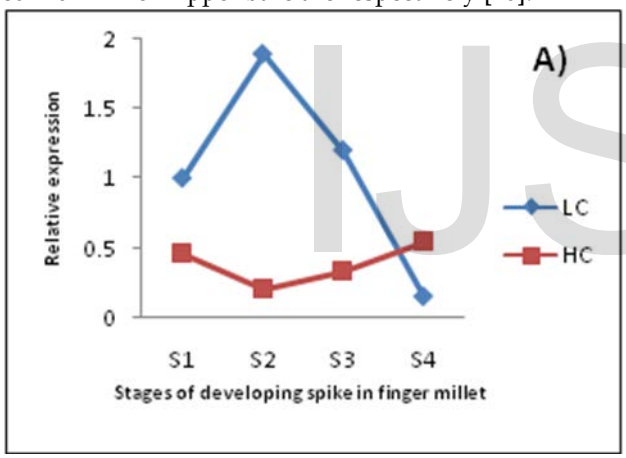

Fig: 2 (A) Relative expression of Ec_CRT1in two different genotypes (HC: high calcum & LC: low calcium) of Finger millet in different developing stages of spike.

From the above result it was clearly seen that there is high expression of *Ec_CRT1* gene in high calcium accumulating genotype (GP-45) as compared to the low calcium accumulating genotype (GP-1) at the later stages of developing spike. The differential regulation of transport machinery is also responsible for the differential calcium ion delivery and spatial distribution in seeds. So, the most plausible explanation of increase in *Ec_CRT1* transcripts in later stages of developing spikes of GP-45 might be due to greater transcriptional synthesis and ultimately the greater translational synthesis which leads to higher accumulation of calcium binding protein in the high calcium accumulating genotype of finger millet. So, this provides a clear evidence that *EcCRT1* probably plays a role in high calcium accumulation through sequestering more calcium in endoplasmic reticulum in later stages of seed development in high calcium containing genotype.

4 CONCLUSION

The results of the present study provide evidence that *Ec_CRT1* is one of the major CaBPs for sequestering and accumulating more calcium during later stages of developing grains of fingermillet in high calcium genotype as compared to low calcium genotype. Thus, further identification and characterization of all other the Calreticulins present in fingermillet will help us to understand how they are involved in the calcium accumulation pathway in developing grains of two contrasting genotypes of fingermillet. Also involving the Molecular cloning of the highly expressed Calreticulin can be fruitful for its functional validation and introgression in another staple crop for biofortification.

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