Genes of calcium transporters

Calcium (Ca^{2+}) is the most plentiful mineral found in the human body. A healthy adult human body contains 1000 -1400 g of calcium, ninety-nine percent (99%) of which is found in the bones and teeth. Nerve cells, body tissues, blood, and other body fluids contain the rest of the calcium. Calcium, a macronutrient essential to human health and well-being performs diverse biological functions in the human body. Calcium is very essential in muscle contraction, oocyte activation, building strong bones and teeth, blood clotting, nerve impulse, transmission, regulating heart beat and fluid balance within cells(Pravina *et al.,* 2013). It serves as a second messenger for nearly every biological process, stabilizes many proteins, and in deficient amounts is associated with a large number diseases and disorders. If calcium levels in the blood drop below normal, calcium will be taken from bone and put into the blood in order to maintain blood calcium levels. Therefore, it is important to consume enough calcium to maintain adequate blood and bone calcium levels. Calcium-rich foods include the dairy group (milk, hard cheese, yogurt, cottage cheese), broccoli, Chinese cabbage (bokchoy), green leafy vegetables (kale, mustard, collards), sardines with bones (canned), dried fruit, nuts and seeds (figs, almonds, soy nuts) and pulses (peas, beans, and lentils). A number of calcium-fortified foods and drinks are also now available, including breakfast bars, cereals, breads, juices, and milk substitutes.

Plants also could be a good alternative source of Ca^{2+}, because some plants exhibit absorbability equal to or better than calcium in milk. Therefore there is an immediate need to explore crop systems with increased abilities to acquire calcium and accumulate them in edible tissues. It is time to

investigate and understand the factors responsible for higher grain calcium accumulation in plants in order to develop calcium biofortified crops. Cereals constitute the family Poaceae (Gramineae) are mankind’s most important source of calories. Continued improvement of Poaceae crops is necessary in order to continue to feed an ever-growing world population. While the importance of such large grained cereal crops as wheat, corn and rice to the beginnings of agriculture are well understood, a small group of small-seeded grasses known as millets are often marginalized or ignored while in terms of proteins, minerals and vitamins, the millets have higher nutritional value than the common cereals of wheat, rice and corn. Millets, small-seeded annuals belonging to family Poaceae were the first crops to be cultivated prior to plough age. Millets rank as the world’s sixth most important food crops among cereals and are primarily grown in Asian and African countries. The millets are categorized as major and minor based on size of seeds and the extent of cultivation. Sorghum and pearl millet are considered as the major millets, while the rest of the millets referred as minor millet which includes foxtail, little, proso, kodo , barnyard and finger millet.

Nature has enormous plant diversity, many of which are unexplored, but they might be unique for a character, finger millet is one of them. Finger millet is a crop rich in Ca^{2+} (upto 450 mg/100g seed), which is about 5-30 times higher than widely consumed cereals like rice, wheat etc, and therefore would be a excellent candidate to study the mechanisms behind higher grain Ca^{2+} accumulation.

Finger millet or Ragi (*Eleusine coracana* L.) is an important crop used for food, forage, and industrial products. Finger millet is considered to be nutritionally rich and is one of the cheapest sources of dietary energy in the form of proteins and carbohydrates. It is an important cereal because of its excellent storage properties of the grains and the nutritive value, which is higher than that of rice and equal to that of wheat. It is also a good source of micronutrients like Calcium, Iron, Phosphorus, Zinc and Potassium which could alleviate the wide spread micronutrient malnutrition in the vulnerable segments in the developing country like India. It is highly rich in calcium and due to the high proportion of fiber, it has hypoglycemic effects which make the products of these crops suitable for consumption by diabetic and heart patients. Moreover this crop has been targeted as nutraceutical crop. High calcium and iron contents make it a suitable for making baby food products. The genotypic differences of seed calcium content might be due to variation in the activities of calcium transport system and calcium sequestering. Such information can help to identify genotypes that are either predisposed to higher calcium accumulation and offer good genetic potential for breeding.

There are three major classes of Ca^{2+} transporter proteins: channels, ATPases (pumps) and exchangers(Smyth *et al.,* 2006). Members of these transporter proteins may differ in their cellular and tissue distribution and the regulation by other signalling pathways(Strehler *et al.,* 2001). The spatial and temporal regulation of calcium concentration in plant cells depends on the coordinated activities of channels and active transporters located on different organelles and membranes(Sze *et al.,* 2000). Calcium channels, pumps and exchangers, which differ in their cellular distribution and

mechanism of transport, operate the complex and tight regulation of calcium homeostasis. Specific isoforms of these proteins are responsible for increasing or reducing free calcium in the cytosol(Monteith *et al.,* 2007). Whereas diffusion of molecules across membranes (either intracellular or across the plasma membrane) is mediated by calcium channels, the calcium pump (ATPase) is a membrane-bound Ca^{2+} transporter that uses energy derived from ATP hydrolysis to transport Ca^{2+} across membranes against their concentration gradient(Nagata *et al.,* 2004). There are two major Ca^{2+} ATPase families(Axelsen *et al.,* 2001): P-type IIA and P-type IIB. The P-type IIA family lacks N-terminal auto-regulatory domain while the IIB family of plant is characterized by the presence of an auto-inhibitory N-terminal domain containing a Ca^{2+} /CaM-binding site and a serine phosphorylation site(Tuteja *et al.,* 2007). Calcium exchanger is a secondary active transporter using energy from the flow of one ion (for example, Na^{+}) down its concentration gradient to transport Ca^{2+} against its concentration gradient(Monteith *et al.,* 2010). Ca^{2+} transporters on various membranes play an important role in orchestrating diverse biological processes. The results of electrophysiological studies and molecular analyses indicate the existence of many species of Ca^{2+} transporter proteins(White *et al.,* 2002).

Ca^{2+} concentration is delicately balanced by the presence of “ Ca^{2+} stores” such as vacuoles, endoplasmic reticulum (ER) and mitochondria. In plants and other eukaryotes, calcium has manifold functions, most prominently as secondary messengers and has roles as a signal carrier. Calcium ions represent a ubiquitous second messenger molecule involved in the

regulation of various metabolic and physiological processes by interacting with a special class of proteins known as calcium binding proteins. These calcium binding proteins involves in Ca^{2+} signalling that decode temporal and spatial changes in cellular Ca^{2+} concentration. Cells usually invest much of their energy to effect changes in Ca^{2+} concentration. Ca^{2+} efflux into the cell exterior and/or the sequestration into cellular organelles such as vacuoles, ER, and mitochondria to restores its level to that of the resting state.

These Calcium-binding proteins form a large family which has been reported to have many functions within the cell, including decoding cellular signals. The CBP can be broadly divided into four major classes: Calmodulin (CaM) (class A), CAM –like and other EF-hand containing calcium binding proteins (class B), Ca regulated protein kinases (class C) and Ca binding proteins without EF hand motifs (class D).

The EF-hand is the most frequent motif found in calcium binding proteins which binds a single Ca^{2+} ion with high affinity. The Ca^{2+} sensors utilize the side-chain oxygen atoms of the EF-hand motif for Ca^{2+} coordination. The Ca^{2+} binding affinities of the EF-hand protein vary substantially and depend on the amino acid sequence of the protein, especially with regard to the 12-residue consensus loop that provides all the acids that directly ligate to Ca^{2+} ions.

Calcium not only plays important roles in mediating cellular signalling, it also accumulates as a principal cation in the grains of many monocots and dicots

during grain filling. This accumulated Ca $^{2+}$ is later used in the activation of many hydrolytic enzymes and signal transduction pathways during germination.

Although much of the research efforts have been directed toward understanding the role of calcium in controlling diverse biological processes, the uptake, assimilation, and accumulation of calcium in developing cereal grains is not clearly understood. The reports published so far suggest that the accumulation of calcium in the seed is governed by both environmental and genetic factors.

The role and mechanism, for preferential accumulation of elements is currently unknown but may be, in part, linked to maximizing elemental availability to the plant. Despite a considerable progress in understanding the calcium signaling and transport in plants, very limited report is available to explain the molecular basis of the higher seed calcium accumulation in plant so far. Thus, a three pronged molecular approach is being used in our laboratory through a merger of approaches like proteomics, functional genomics and molecular marker technology to investigate the translocation and accumulation of calcium from source to sink in plants. Association mapping studies in finger millet identified three closely associated markers for calcium accumulation (Unpublished data). Studies in our lab focused on how does high amount of calcium get accumulated in finger millet grains and revealed that calcium is bound to calcium binding proteins and is chiefly deposited in the aleurone layer(Nath *et al* ., 2012).

Further, two calcium binding proteins ‘ Calcinurin-B’ and ‘ Calreticulin’ were identified for the first time in the finger millet grains employing several techniques including peptide mass fingerprinting which might be involved in high grain calcium accumulation. The differential spatial distribution of grain calcium was investigated through defining the roles of calcium signaling and transporter machinery. Molecular cloning and comparative *in silico* analysis of calmodulin genes from cereals and millets were done for understanding the mechanism of differential calcium accumulation(Nath *et al* ., 2010). Since, complete genomic information of finger millet is not available at present partial mRNA sequences of 7 gene(s) encoding calcium sensors and transporters (CaM-01 gene, CaMK-02 gene, CaATPase-01 gene, CaX-01 gene, TPC-01 gene, and one gene of 14. 3. 3) of finger millet were isolated using conserved primer approach. Of these genes, CaM, and CAX1 were strongly expressed in the late stages of spike development and thus might be responsible for accumulating high concentrations of calcium in finger millet seeds(Mirza *et al.,* 2012). Immunodetection analysis revealed that CaM is localized in the embryo and close to the aleurone layer and accumulates in higher amounts in the high grain calcium genotype. It is indicated that abundance of CaM around aleurone layer might activate calcium ATPase and thereby responsible for high grain calcium accumulation. Southern hybridization have shown the presence of at least 4 copies of CaM gene that might be located on different regions of the finger millet “ AABB” genome and be a reason of its transcripts abundance(Kumar *et al.,* 2013).

Calcium accumulation in plant is a complex trait and depends on both genetic and epigenetic factors, so need genome wide study of calcium

transporters and calcium binding genes. Currently, finger millet has only 1956 EST and 818 nucleotide sequences available in NCBI database. The conventional methods of gene cloning and sequencing are not only time consuming and expensive but also yield only limited amount of genetic information. Hence RNA-seq has provided a powerful tool for analysis of transcriptomes. For non-model organisms with limited genomic information, transcriptome sequencing provides a cost-saving tool by only sequencing functional and protein coding RNAs, thus providing direct information about the genes. In the absence of a completely sequenced genome such as finger millet transcriptomic resources for non-model species by next generation sequencing (NGS) have proven extremely useful for plant research and seed development process. For non-model organisms like finger millet, genome-wide information that describes functionally relevant variation may be obtained by RNA-Seq following *de novo* transcriptome assembly. These sequencing strategies does not need prior knowledge of gene sequences thus allowing de novo assembly of transcripts in non model species that lack a reference genome and discovery of noval genes and expression profiles under specific condition.

It can be speculated that the accumulation of calcium in the developing grains could be the result of extensive calcium signaling with the involvement of calcium transporters. In order to understand the accumulation and distribution of calcium inside the seed we recently found that in the developing finger millet grains, calcium is mainly deposited in the aleurone layer of the seed leading to an assumption that there may be specific calcium transporters operative in the seed to carry out such

differential spatial distribution. Since, calcium is an important macro nutrient in human diet and the accumulation of calcium in some cereal grains like finger millet is high, it is necessary to understand the molecular basis of high grain calcium in cereals grains. Such a study will enable us to understand and formulate effective strategies to increase the levels of calcium in grains. Therefore, in the parallel studies on rice, sorghum, and finger millet (high & low calcium variety), have been studied to identify possible candidate genes of calcium transporters and calcium binding proteins across different stages of seed that may be responsible for the accumulation of calcium in the developing grain. Such a combined approach of *in silico* analyses and experimental follow-up will lead to comprehensive understanding of the crucial events of seed development in cereal grains. Furthermore the genes identified to be up-regulated in the high grain calcium content genotype can be used as a candidate gene for improving the calcium content of *Eleusine* genotypes which are agronomically better performers but contain low calcium. This would not only help in understanding the mechanism of calcium accumulation in finger millet but would also open newer vistas in nutritional enrichment of crops while still retaining agronomically favorable characteristics.

The present investigation was carried out to identify potential candidate genes that may be responsible for the accumulation and storage of calcium in the developing grain in finger millet with the following objectives:

1. To identify potential genes of calcium transporters from *Oryza sativa* and *Sorghum bicolor* using bioinformatics tools in order to understand

the calcium accumulation during the grain filling in *Eleusine coracana* (finger millet)

2. To characterize the expression patterns of calcium transporters involved in the spatio-temporal distribution and accumulation of calcium using rice MPSS and affimetrix microarray data to corroborate the information in *Eleusine coracana* .
3. To validate the expression patterns of up-regulated calcium transporter genes in rice, sorghum and finger millet through quantitative-reverse transcription polymerase chain reaction (Q-PCR) method.
4. To analyze and characterize the seed specific calcium binding proteins from the available genome sequence information of C3-rice and C4-sorghum using *in silico* biology.