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Short Communications

Identification and characterization of rice ortholog of *ferric chelate reductase* (*FRO2*) gene in little millet (*Panicum sumatrense* Roth ex Roem. & Shult.)

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In this study, a rice ortholog of *ferric chelate reductase* (*FRO2*) gene, involved in plant metal uptake phenomenon, has been identified and characterized in high iron containing small millet. The work has been accomplished in little millet (*Panicum sumatrense* Roth ex Roem. & Shult.) using PCR based amplification and next generation sequencing technology. The genotype RLM-37 was used to amplify *FRO2* gene, followed by its sequencing on Ion Torrent's sequencing technology. Gene specific primers designed from rice genome, to amplify full-length gene fragment of 2.8 Kb in rice, generated an amplicon of 2.7 Kb in RLM-37 and its sequencing generated a sequence of 2691 bp. The sequenced amplicon showed high-level sequence similarity with Os*FRO2* gene at nucleotide level, whereas low-level sequence homology and structural similarity was observed at protein level in the database. The predicted structure of the gene showed the presence of 6 exons and 5 introns in the little millet gene. The predicted protein sequence domain search showed the presence of *ferric reductase* domain and *NOX_Duox_Like_FAD_NADP* domain. The NOX_Duox_Like_FAD_NADP domain is unique to this protein, covering 36% of the millet protein. The 3D structure of this domain was elucidated by homology modelling method and the catalytic domain part was characterized. Thus, the study provides the start point for gene discovery in underexplored millet crops for improving grain nutritive traits.

Keywords: Amplicon sequencing, gene ortholog, homology, little millet, metal homeostasis

Minor millets are underutilized coarse cereal crops. These can be grown in the extremes of climatic conditions where most other cereal crops may not survive to produce grain. Minor millets are staple food for the tribal people where cultivation of major cereals like rice, wheat and maize is not popular[1,2]. Screening of minor millets for grain nutritive traits by several workers has shown that these millets are highly nutritious food crops with higher fiber content along with quality protein and mineral composition, which can serve as excellent dietary source for these elements[3]. They bear potentials to serve as future food to combat the deep rooted malnutrition and nutritional insecurity prevailing in the developing world[4,5]. Studies also reveal the existence of great variability among the collection of millets for grain nutritive traits, which can be exploited by employing efficient breeding strategies to improve these traits. Along with these facts, millets identified with high nutritive values can also be exploited for the identification and mining of genes/alleles/genomic regions governing these traits. Little millet (*Panicum sumatrense* Roth ex Roem. & Shult.), locally known as *Kutaki*, is popularly consumed by tribals of Chhattisgarh, India and bear health benefits in terms of high iron, zinc and protein content. Preliminary screening for grain micronutrient contents in different varieties of minor millets at the Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur has shown considerably fair amounts of Fe and Zn in the millet grains.

Cereals are important source of food worldwide but, unfortunately, they contain very low levels of bioavailable micronutrients, such as, iron and zinc, for a balanced human diet. This leads to micronutrient malnutrition in the population, which is a serious global health challenge[6,7]. Thus human intake of such nutritional elements depends on their concentration in the edible portion of plants and their bioavailability. Furthermore, it is known that plants maintain metal ion homeostasis through sophisticated mechanisms of the acquisition and distribution of metal ions to the specific compartments and for storage. Understanding the molecular mechanisms underlying this phenomenon and the interplay of genes involved is the key to improve metal ion concentration in the edible plant parts. Low availability of iron often limits plant growth because iron forms insoluble ferric oxides, leaving only a small, organically complex fraction in soil solutions. The enzyme ferric chelate reductase is required for most plants to acquire soluble

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iron. It is a membrane bound protein involved in reduction of Fe (III) to Fe (II), in strategy I of iron uptake[8]. Strategy I, mainly adapted to dicotyledoneous and non-graminaceous monocotyledoneous species, is a reduction based strategy to convert ferric form of iron to readily available ferrous form. Studies on model cereal-genome rice have shown high association of the gene encoding this enzyme with the grain loading of iron and zinc, especially, at mid grain filling stage[9]. Thus, the food crops rich in iron like millets may serve as reservoirs of new genes, alleles *etc.* involved in efficient Fe uptake, transport, remobilization and grain loading. As millets lack enriched genomic databases, the knowledge from the reference model genomes can be exploited and employed to search for metal homeostasis related gene orthologs in minor millet crops. The present study involves estimation of Fe and Zn contents in the millet grains, followed by various molecular biology techniques for gene ortholog identification.

Whole grain of millets was dehusked manually using sand paper prior to estimation of micronutrients. Fe and Zn concentration were estimated as per *HarvestPlus*[10] guidelines using atomic absorption spectrophotometer (AAS200) and tomato leaf powder as standard[8]. Iron and zinc content of over 30 ppm were estimated in different minor millet accessions including little millet genotype RLM- 37, which carries 32 ppm Fe and 32.4 ppm of grain Zn levels. This genotype also bears fairly good amount of protein and essential amino acids (Table 1).

Sequence for the rice gene Os*FRO2* was retrieved from rice genome browser (rice.plantbiology.msu.edu /cgi-bin/gbrowse/rice) for the reference genome *Nipponbare* and genomic DNA based primers were designed from this sequence to amplify full length gene fragment. Long range PCR was performed using this primer set on little millet (RLM-37), which generated an amplicon of 2.7 kb (Fig. 1). Same primer set generated short amplicons in other millets tested (different genotypes of Barnyard millet), whereas rice sample generated an expected fragment of 2.8 kb. Amplified fragment was gel purified using Sigma's gel elution kit (GenElute Gel Extraction Kit) and purified DNA sample of little millet (RML-37) was processed for sequencing. Sequencing was performed on Life Technology's Personal Genome Machine (PGM) employing *Amplicon Sequencing* method based on Ion Torrent technology following manufacturer's guidelines for every procedure (www.lifetechnologies.com). 200 bp read libraries were prepared, which generated 2.2 million reads. Sequenced reads thus obtained were assembled using Assembler Plug In (ioncommunity.lifetechnologies.com). As a result, a sequence of 2691 bp was obtained for *FRO2* gene from little millet. The obtained sequence was subjected to analysis using BLASTn algorithm available at http://www.ncbi.nlm.nih.gov. This showed high level sequence similarity with *OsFRO2* gene at nucleotide level with 100% similarity, 0 expect value over 85% query coverage. Further, the predicted structure of the gene was worked out by FGENESH gene prediction tool (www.softberry.com), which showed the presence of 6 exons and 5 introns. Start and end positions of the exons and introns, poly A site *etc* are shown in Fig. 2.

The amino acid sequence was deduced *via* nucleotide sequence translation method of EMBOSS transeq tool available at www.ebi.ac.uk/Tools/st/ from isolated

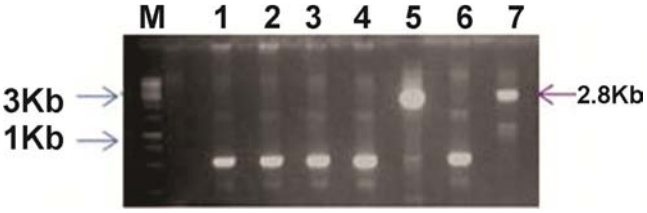

Fig. 1—PCR amplification of full length *FRO2* gene fragment in different minor millets and rice samples. [M: 1 kb DNA ladder; 1, 2, 3, 4, 6: Different Barnyard millet genotypes, namely, Melaghat 1, Melaghat 2, Melaghat 3, Melaghat 4 and Sawa Local, respectively; 5: Little millet genotype RLM-37; 7: IRX HMT (Rice)].

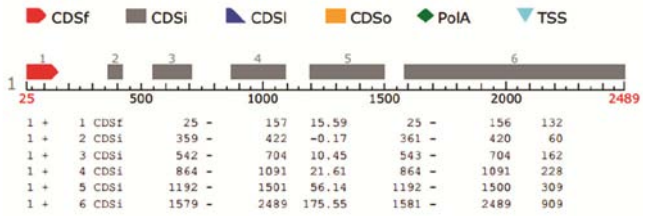

Fig. 2—Predicted gene structure of *FRO2* from little millet as analyzed by FGENESH gene prediction tool.

Table 1—Nutritional profile of little millet (*P. sumatrense*) genotype selected for the study

Genotype	Collection	Nutritional profile (full grains)				
		Fe (ppm)	Zn (ppm)	Protein (%)	Tryptophan (g/g)	Lysine (g/g)
RLM-37	Jagdalpur (Chhattisgarh)	32.00	32.40	10.30	0.09/16 N	1.92/16 N

nucleotide sequence of little millet. The protein sequence was subjected for similarity search against protein data bank (PDB) (http://www.rcsb.org/pdb/home/home.do). The result shows that little millet protein sequence have low level sequence similarity with minimum coverage with respect to available protein structures. Moreover, the protein sequence was submitted for the identification of conserve domain in conserve domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Conserved domain analysis found two domains, (a) Ferric reductase like transmembrane component (52-136), and (b) *NOX_Duox_Like_FAD_NADP* (166-354), which catalyzes the generation of reactive oxygen species (ROS), such as, hydrogen peroxide and superoxide (Fig. 3). Ferric reductase family is a family of flavocytochromes capable of moving electrons across the plasma membrane. A common feature of this superfamily is the high similarity around the FAD, NADPH and heme binding sites[11]. A heme-containing transmembrane ferric reductase domain (FRD) is found in bacterial and eukaryotic protein families, including ferric reductases and NADPH oxidases (NOX). Ferric reductase domain is known to transfer electrons from cytosolic NADPH to extracellular ferric ions to generate the reduced form of ferrous ions, which can then be transported across the plasma membrane by specific iron transporters[12]. Looking into the rice gene *OsFRO2*, the same conserved domains (Ferric reductase & *NOX_Duox_Like_FAD_NADP*) were found to be present with variation in the span of these domains in comparison to the millet.

In order to have an initial idea on the structure of NOX_Duox_Like_FAD_NADP domain of little millet, homology modeling based structure prediction was performed using Swiss Modeler. The analysis was done on the basis of coverage and identity with the probable template based on BLAST search against PDB. In this case, the best template was chloroplast Ferredoxin-NADP$^+$ oxidoreductase of *Capsicum annuum* Yolo Wonder (paprika) (PDB id: 1sm4). Sequence alignment with this template has shown higher similarity with query coverage between 166-357 amino acids (Fig. 4a). The domain fragment was found to be 36% of the total little millet protein sequence length. This domain of the protein catalyzes the generation of ROS and contains 9 beta sheets, two helix with flavin adenine dinucliotide (FAD) binding domain (Fig. 4 b). FDA helps NOX to catalyze the generation of ROS. However, the 3D structure of ferric reductase could not be obtained based on homology modeling as the templates was not found in PDB.

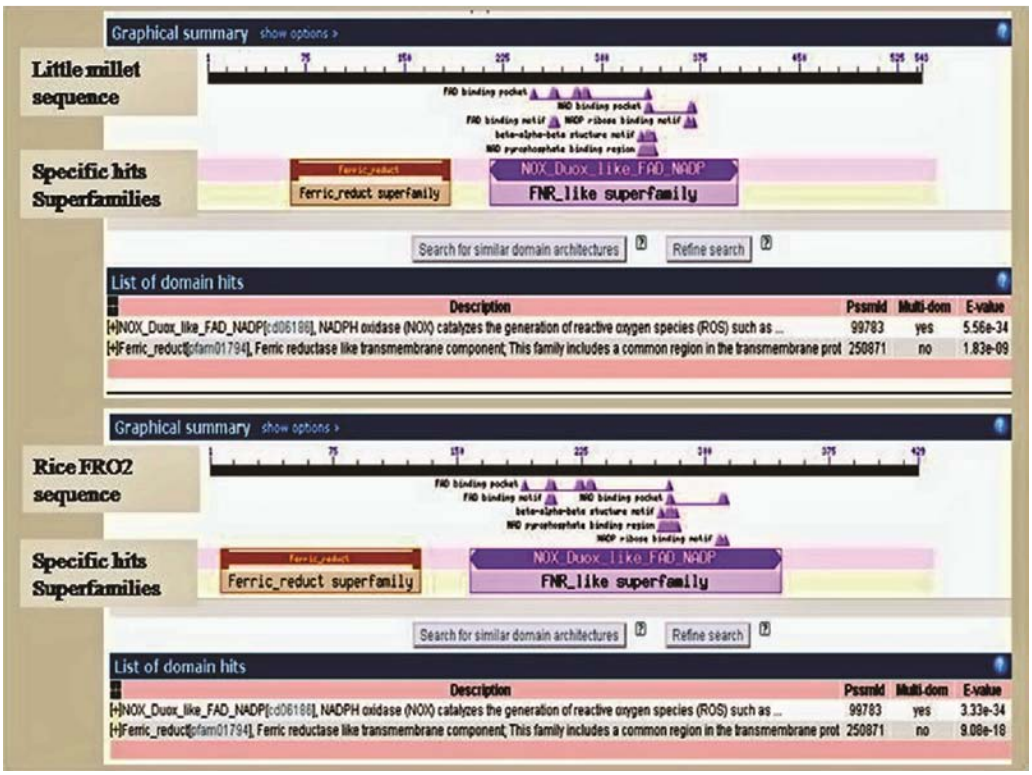

Fig. 3—Conserve domains found to be present in the sequence of little millet in comparison to the rice *FRO2* gene sequence.

Fig 4 (a & b)—(a) Alignment of target sequence (little millet protein sequence) with chloroplast ferredoxin-NADP$^+$ oxidoreductase protein sequence (PDB id: 1sm4) of *C. annuum*; (b) Ribbon diagram of catalytic domains of NOX_Duox_Like_FAD_NADP of little millet protein.

Homology based modeling of NOX_Duox_Like_ FAD_NADP provided an initial idea of basic structure of little millet protein. However, in order to have a detailed information and authentication of the findings on full-length protein structure, an alternative method of protein modeling, *i.e.*, threading based protein modeling method, followed by molecular dynamic simulation, would be employed to get stable and full protein structure of little millet. Through the present work, an ortholog of rice *FRO2* gene has been identified in little millet (genotype RLM-37) using PCR based amplification and next generation sequencing technology. Moreover, a baseline data has been generated for the discovery of metal homeostasis genes in millet crops. Valuables genes or their orthologs identified would serve as new sources and targets for manipulation of improving grain micronutrient levels. They would also have implications for the generation of crops with improved nutritional quality and increased growth in nutrient deficient soils.

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