Identification and characterization of a grain micronutrient-related *OsFRO2* rice gene ortholog from micronutrient-rich little millet (*Panicum sumatrense*)

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Abstract Minor millets are considered as nutrient-rich cereals having significant effect in improving human health. In this study, a rice ortholog of Ferric Chelate Reductase (*FRO2*) gene involved in plant metal uptake has been identified in iron-rich Little millet (LM) using PCR and next generation sequencing-based strategy. *FRO2* gene-specific primers designed from rice genome amplified 2.7 Kb fragment in LM genotype RLM-37. Computational genomics analyses of the sequenced amplicon showed high level sequence similarity with rice *OsFRO2* gene. The predicted gene structure showed the presence of 6 exons and 5 introns and its protein sequence was found to contain *ferric reductase* and *NOX_Duox_Like_FAD_NADP* domains. Further, 3D structure analysis of FCR-LM model protein (494 amino acids) shows that it has 18 helices, 10 beta sheets, 10 strands, 41 beta turn and 5 gamma turn with slight deviation from the

FCR-Os structure. Besides, the structures of FCR-LM and FCR-Os were modelled followed by molecular dynamics simulations. The overall study revealed both sequence and structural similarity between the identified gene and *OsFRO2*. Thus, a putative ferric chelate reductase gene has been identified in LM paving the way for using this approach for identification of orthologs of other metal genes from millets. This also facilitates mining of effective alleles of known genes for improvement of staple crops like rice.

Keywords Sequencing · Ferric Chelate Reductase · Little millet · Metal homeostasis · MD simulation

Abbreviations

LM	Little millet
Fe	Iron
FCR-LM	Ferric chelate reductase-Little millet
FCR-Os	Ferric chelate reductase- <i>Oryza sativa</i>
FRO2	Ferric reduction oxidase 2
PCR	Polymerase chain reaction
NGS	Next generation sequencing
Zn	Zinc
MD	Molecular Dynamics

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Introduction

Minor millets are underutilized coarse cereal crops which can be grown in the extremes of climatic conditions, where most other cereal crops may not thrive to produce grains. Minor millets form staple food for the poor people of tribal areas where cultivation of major cereal crops like rice, wheat and maize is not popular (Chopra and Neelam 2004; Desai et al. 2010). Studies by various researchers reported

that minor millets are nutritious food crops and contain ample amount of fibers, proteins and minerals. These composition nutrients make them essential element of dietary foods (Amadou et al. 2013). They serve as potential future food with capability of erasing the deep rooted malnutrition and thus avert the nutritional insecurity prevalent in the developing world (Singh and Raghuvanshi 2012; Saleh et al. 2013). Studies also reveal great variability existing among the collection of millets for grain nutritive traits which can be exploited by employing efficient breeding strategies to improve these traits. Along with this, millets varieties and genotypes containing high nutritive values can also be made use of for the excavation of genes/alleles and genomic loci governing these traits. Among different millets, Little millet (*Panicum sumatrense*) locally known as “Kutaki” is popularly consumed by tribal's of Chhattisgarh and bear health benefits in terms of high iron, zinc and protein content. Preliminary screening for grain micronutrient contents of different varieties of minor millets at Department of Plant Molecular Biology and Biotechnology, IGKV, Raipur has shown considerably fair amounts of Fe and Zn in millet grains (Chandel et al. 2014, 2016).

Cereals are important source of food across world but their nutritional value is often limited by very low levels of bio-available micronutrients (such as iron and zinc) for a balanced human diet leading to micronutrient deficiency driven malnutrition in such population resulting in a serious global health challenge (Graham et al. 2001). Furthermore, plants are known to maintain metal ion homeostasis through sophisticated mechanisms, which firmly control the acquisition and distribution of metal ions to the specific compartments and storage. Recently used plant genomic approaches made possible to do fast identification of molecular components related to mineral homeostasis. Using genomics approaches 39 metal homeostasis-related genes along with four well-characterized homologs have been identified in rice genome (Gupta et al. 2017; Gross et al. 2003). The low availability of iron among different minerals often leads to inhibit the plant growth. This is due to the formation of insoluble ferric oxides in the presence of oxygen leaving small fraction of Fe in the soil solutions. The enzyme ferric chelate reductase is prerequisite for most plants to acquire soluble iron. It is a membrane-bound protein involved in reduction of Fe(III) to Fe(II) in strategy I of metal uptake. The genes encoding enzyme *AtFRO1*, *AtFRO2* have been isolated and characterized from roots of *Arabidopsis* under Fe-deficient condition. These genes belong to a super family of flavocytochromes and involved in transporting electrons across the membrane (Narayanan et al. 2007; Robinson et al. 1999). Studies on rice genome have shown high association of gene encoding this enzyme with the grain loading of iron

and zinc especially at mid grain filling stage (Narayanan et al. 2007). Thus, high iron-rich food crops like millets may serve as reservoirs of new genes, alleles, etc. involved in efficient Fe uptake, transport, reallocation to various organs 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 (Ross and Robin 2004; Chandel et al. 2010). New sequencing platforms have made easy and fast gene and genome sequencing for most of the organism including crop plants. Therefore, in this study an attempt has been made to identify and characterize rice orthologs of ferric chelate reductase (*FRO2*) gene involved in plant metal uptake phenomenon in high iron containing small millet using PCR and next generation sequencing approach. The identified sequence in LM has been further validated by ascertaining its correspondence and relevance to the *OsFRO2* gene of rice (taken as reference in the study). This has been established more efficiently through comprehensive sequence analysis, homology searching and functional domain identification along with protein modeling and Molecular Dynamics (MD) simulation study.

Materials and methods

Sample preparation and grain micronutrient estimation

Whole grains of LM genotype RLM-37 were dehusked manually using sand paper prior to estimation of micronutrients. Fe and Zn concentration were estimated as per HarvestPlus guidelines (www.harvesplus.org) using Atomic absorption spectrophotometer (AAS200) considering tomato leaf powder as standard (HarvestPlusI 2006). It was also subjected to grain protein and amino acid analysis as per the method described by Johri et al. 2000 to assess its nutritive value.

Amplification and sequencing of full length gene sequence of *OsFRO2* in minor millets

The sequence of rice gene *OsFRO2* available for reference rice genotype Nipponbare was retrieved from rice genome browser (<http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice>). The gene sequence-based primers were designed to amplify full length gene sequence. Long range PCR was performed using primer set on RLM-37 to generate full length gene sequence. Same primer set was also utilized to check its amplification potential in rice DNA sample included as control template in PCR assay. The amplified fragments were gel purified using gel elution kit (GenElute

Gel Extraction Kit, Sigma, USA) and the purified DNA samples were processed for sequencing. The sequencing was performed on Life Technology's Personal Genome Machine (PGM) employing *AmpliSeq* method based on Ion Torrent technology following manufacturer's guidelines (www.lifetechnologies.com). Two hundred bp DNA fragment size libraries were prepared for sequencing. Sequenced short reads thus obtained were assembled using assembler plug in (<http://ioncommunity.lifetechnologies.com>) to obtain the complete sequence for *FRO2* gene from LM.

Bioinformatics analysis of sequenced amplicon

The finished *OsFRO2* gene sequences obtained after assembly were subjected to homology search analysis using BLASTn algorithm available at <http://www.ncbi.nlm.nih.gov> to analyze its similarity with the reference rice gene sequence and other potentially similar sequences available for crop plants in the database. The predicted structure of the gene was obtained by FGENESH gene prediction tool (www.softberry.com). Further, the amino acid sequence was deduced via nucleotide sequence translation method of EMBOSS Transeq tool available at www.ebi.ac.uk/Tools/st/ from the isolated nucleotide sequence of LM. The protein sequence was then subjected for similarity search against protein data bank (PDB) (<http://www.rcsb.org/pdb/home/home.do>) and conserved domain database (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) for the identification of conserved domains.

Protein modelling

The protein modelling was carried out for FCR-LM and FCR-Os protein sequences. Both sequences were submitted in DELTA-BLAST for identification of templates in PDB (Boratyn et al. 2012). The BLAST result showed low sequence similarity for FCR-LM and FCR-Os with the available protein templates. The sequence identity of FCR-LM and FCR-Os with best identified template was 17.42 and 17%, respectively, whereas the query-coverage for FCR-LM and FCR-Os was 52 and 53%, respectively. Due to unavailability of perfect homologous template in PDB, the homology modeling for these protein sequences was not possible and this made us to perform modeling of both the protein structures through threading method using I-TASSER server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The server generates 5 threading models using different programs and the best model was selected based on minimum C score (Yang 2008). The secondary structural features of the sequences were generated by ProFunc

Server (<http://www.ebi.ac.uk/thornton-srv/databases/profunc/>). The structural validation of the best models was performed using SAVES server (<http://nihserver.mbi.ucla.edu/SAVES/>). Finally the validated models were submitted for all atom MD simulation.

Molecular dynamics simulation of FCR-LM and FCR-Os

All atom MD simulations with similar parameters and ambient environmental conditions were applied for both the models. AMBER99SB force field was applied on the system of FCR-LM and FCR-Os models using GROMACS software platform (Berk et al. 2008). Simple point charge (SPC) water model was used to solvate the system and 2 ferro second (fs) time step was used for simulation (Berendsen et al. 1981). The neighbours search list was updated in every 10 fs using grid method, which has a cut off radius of 1 nano-meter (nm). In every 2 picoseconds (ps), the coordinates of all atoms were updated in the cubic simulation box. The required Sodium ions were added for neutralizing the acidic environment of the box. In this, system first Canonical ensemble (NVT) ensemble was applied with normal temperature (300 K, $\tau_p = 1$ ps) using Berendsen thermostats method and later on Isothermal-isobaric (NPT) ensemble was applied with normal pressure ($P_0 = 1$ bar, $\tau_p = 1$ ps) using Parrinello-Rahman baro stats pressure coupling method (Berendsen et al. 1984; Roy et al. 2012). The Particle Mesh Ewald (PME) algorithm was used for electrostatic term computation. The system energy was first optimized with the "steepest descent minimization" algorithm and then by using the "conjugate gradients" algorithm. The production run period was set for 15 nano second (ns) and the generated trajectory was analyzed using gromacs-associated programs, viz., *g_enegy*, *g_rmsd*, *g_rmsf*, *g_cover*. All figures were created by USF chimera visualization software (<https://www.cgl.ucsf.edu/chimera/>).

Results and discussion

Grain nutritive value in LM genotype

Elemental analysis for micronutrient estimation revealed that Iron and Zinc content of over 30 $\mu\text{g/g}$ were found to be present in LM genotype RLM-37. It contained 32 $\mu\text{g/g}$ Fe and 32.4 $\mu\text{g/g}$ of grain Zn levels. Analyzing this genotype for other nutritive traits showed that RLM-37 also had fairly good amount of protein and essential amino acids (Table 1).

Table 1 Nutritional profile of Little Millet (*Panicum sumatrense*) genotype selected for the study

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

Characterization and structural/functional annotation of full length gene sequence of FCR-LM

The gene-based primers targeting the reference rice gene *OsFRO2* were designed and were tested for amplification on genomic DNA of LM genotype RLM-37. This resulted in amplification of 2.7 Kb gene fragment in LM which was in correspondence to the expected fragment size generated in a control rice sample (Fig. 1). The same primer set was observed to produce short amplicons in other millets tested (different genotypes of Barnyard millet). The full length gene amplicon sequencing in next generation sequencer (Ion Torrent's Personal Genome Machine—Life technologies) generated 2691 bp of *FRO2* gene sequence from LM (see Supplementary Material). BLAST sequence homology search of this 2691 gene amplicon sequence revealed high level sequence similarity with *OsFRO2* gene at nucleotide level with 100% similarity, 0 expect value over 85% query coverage. Further, downstream analysis of *FRO2* gene sequence from millet using in silico tools for structural and functional characterization showed the presence of structural features and protein domains unique to ferric reductase family of genes. In detail, structural annotation exhibited the presence of 6 exons and 5 introns (Figure S1a) which is same as found in rice *OsFRO2* gene sequence. The identified gene sequence has been deposited to Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) as Ferric chelate reductase-Little millet and the allotted Acc. No. is KY523105. In addition to this, primer sequences

were designed using sequenced amplicon as query and were used to amplify the corresponding fragments in different millets (Table 2). Two millet sequence derived primer sequences were able to amplify the expected size of PCR products in RLM-37 thus validating the amplicon and primer sequences (Fig. 1).

The protein sequence of LM was then subjected to similarity search against protein databank that showed low level sequence similarity, with minimum coverage, with respect to the available protein structures. Moreover, the protein sequence was submitted for the identification of conserved domain(s) in conserved domain database (CDD) to identify the conserved domain(s). The conserved domain analysis found two domains: (a) Ferric reductase like transmembrane component (52–136) a family of flavocytochromes capable of moving electrons across the plasma membrane and (b) NOX_Duox_Like_FAD_NADP (166–354), which catalyzes the generation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide (Figure S1b) (Gupta et al. 2016).

Modeling and structural validation

The 3D models of FCR-LM and FCR-Os were modeled via threading method using I-TASSAR server. Five models, each for FCR-LM and FR-Os were generated using ten different templates reported by 10 different threading programs (Essman et al. 1995). The structural coordinates of the selected templates covered different parts of the query sequence. Sequence alignment of FCR-LM and FCR-Os with the selected 10 templates having different identity scores, query coverage scores and Z scores were predicted in Figure S2 and S3, respectively. Only one best model of FCR-LM (C-Score = −2.36) and FCR-Os (C-Score = −1.83) were selected out of predicted 5 models or templates for structural validation and further consideration. The selected 3D model of FCR-LM consisted of 494 residues forming 18 helices, 10 beta sheets, 10 strands, 41 beta turn and 5 gamma turns, while the selected FCR-Os had 489 residues forming 19 Helices, 11 beta sheets, 11 strands, 45 beta turns and 4 gamma turns (Figs. 2a, 3a) identified by ProFunc server (Laskowski et al. 2005). Moreover, the conserved domains in both the proteins were also identified using Conserved Domain Database. Proteins contained the aforementioned two domains: (a) *Ferric reductase* like transmembrane component-a family of

Fig. 1 PCR amplification of full length *FRO2* gene fragment in different minor millets. *M* 1 Kb DNA ladder; 1, 4, 7; Little millet (RLM-37); 2, 5, 8 Barnyard millet (Sawa local) 3, 6, 9 Finger millet (GPU67) using different primer sets detailed in Table 2

Table 2 Details of primer sequences used to amplify ferric chelate reductase gene and generated fragments in little millet

Primer set	Source	Sequence (5' → 3')	Tm	Expected PCR product size	Generated fragment size in little millet
1	<i>OsFRO2</i> gene (rice)	F: GTGTGACTTGTGTCCCAGTG R: CCTTGTCTCCAAACCCATC	59 °C	2880 bp	2691 bp
2	Sequenced amplicon in Little millet	F: CTCCCACACAAATTCACCTAC R: AGCAACATCGTTGCACAGAC	62 °C	2310 bp	2300 bp ^a
3	Sequenced amplicon in Little millet	F: GTACTGGGGTCCAAAGTCAGAG R: GTCGACCATGAAGAGGAACAC	61 °C	919 bp	900 bp ^a

^a The indicated size is approximate fragment size as observed on 1.5% agarose gel

Fig. 2 **a** 3D structure of FCR-LM generated through threading-based method with different colored domains and regions. The N-terminal domain (residues 1–51; *Orange red*), Ferric reductase domain (residues 52–136; *Cyan*), linker helix (residues 137–165; *Violet color*), Nox_Duox_Like_FAD domain (165–355; *blue*) and

Helix+ C-Terminal domain (residues 366–494; *Forest Green*). **b** Secondary structure representation for FCR-LM model generated via ProFunc Server. **c** Structural comparison of residues of FCR-LM with respective residues of X-ray and NMR structures made using PROSA web server

flavocytochromes (b) *NOX_Duox_Like_FAD_NADP*. Detailed description of the structure and different domains of FCR-LM and FCR-Os are depicted in Fig. 2b, 3b, respectively. The structural validation statistics was predicted by SAVES server, which includes different

programs for the calculation of sequential and structural features of the protein (Table 3). Ramachandran's plot meant for validation of the predicted structure, were generated via PROCHECK program to predict the presence of residues in different regions (Laskowski et al. 1993). The

Fig. 3 **a** 3D structure of FCR-Os generated through threading-based method with different colored domains and regions. The N-terminal domain (residues 1–66; *Orange red*), Ferric reductase domain (residues 67–188; *Cyan*), linker helix (residues 189–217; *Violet color*), *Nox_Duox_Like_FAD* domain (218–406; *blue*) and Helix+ C-

Terminal domain (residues 407–489; *Forest Green*). **b** Secondary structure representation for FCR-Os model generated via ProFunc Server. **c** Structural comparison of residues of FCR-Os with respective residues of X-ray and NMR structures made using PROSA web server

allowed regions for FCR-LM and FCR-Os showed 98.0 and 97.9% residues respectively, while remaining % residues are present in disallowed regions (Table 3). Plot statistics confirmed that the maximum numbers of residues for both the structures were well modeled accurately. Evaluation of FCR-LM and FCR-Os with ProSA-Web revealed the Z score values as -5.32 and -4.66 , respectively. This showed that both structures have native features and near to crystal structure (Figs. 2c, 3c). ERRAT calculated the overall quality factor scores for non-bonded atomic interaction and scored to the tune of 32.099 and 30.977 in FCR-LM and FCR-Os, respectively (Prasad et al. 2013, b). These low scores revealed that in both the structures non-bonded atomic interactions were not up to the mark. The

compatibility of amino acids in 3D for both the structures was identified by VERIFY-3D program. Result revealed that 66.87 and 72.60% residues of FCR-LM and FCR-Os were found in 3D. Such high percentage values were quite acceptable, but yet the structures need improvement. Overall quality G-factor for FCR-LM and FCR-Os were found to be -0.47 and -0.50 , respectively, which reveals the dihedral angle position of residues in structure (Prasad et al. 2012). The validation statistics and structural features of both the structures show that there is a need for further improvement in the structure. Hence, energy minimization in ambient environment conditions was performed and then relaxed the structures in water solution using MD simulation tool.

Table 3 Validation statistics of the predicted protein models of FCR-LM and FCR-Os prior to and after molecular dynamics simulation

Structure validation statistics	FCR-LM (%)	FCR-Os (%)	FCR-LMm (%)	FCR-Osm (%)
PROCHECK score				
% Amino acid in most favored regions	83.3	81.9	86.0	86.5
% Amino acid in additional allowed regions	11.0	11.6	12.6	10.9
% Amino acids in generously allowed regions	3.7	4.4	1.1	1.2
% Amino acids in disallowed regions	2.0	2.1	0.2	1.4
PROSA Z score	−5.32	−4.66	−5.37	−3.73
ERRAT score	94.142	30.977	92.352	88.256
VERIFY 3D score	88.03	72.6	87.07	82.65
Overall G factor	−0.47	−0.50	−3.60	−4.54

The Ferric chelate reductase-Little millet (FCR-LM), Ferric chelate reductase-Little millet after MD simulation (FCR-LMm), Ferric chelate reductase-*Oryza sativa* (FCR-Os) and Ferric chelate reductase-*Oryza sativa* after MD simulation (FCR-Osm)

Elucidation of structures of FCR-LM and FCR-Os through MD Simulation

Initially, the structures of FCR-LM and FCR-Os were energy minimized in normal temperature and pressure. Later on, the total energy of each structure was conserved and resulted in stable confirmation. The stable confirmations of FCR-LM and FCR-Os were further equilibrated in the presence of SPC water model and relaxed for 15 ns. The total energy profile (Fig. 4a) for FCR-LM and FCR-Os proteins were found to be negative throughout the

simulation. The average total energy of FCR-LM and FCR-Os was −1114.870 and −907.979 kJ/mol, respectively. The average total energy of FCR-LM protein showed more stability in comparison to FCR-Os. The calculated root mean square deviation (RMSD) plot of simulated structures of FCR-LM and FCR-Os are given in Fig. 4b (Gupta et al. 2015a, b). The backbone deviation was calculated with respect to initial protein structures. The deviation of FCR-LM protein was increased up to 5.66 ns and found to be of 4.56 Å deviations. After this point, the structure of FCR-LM tried to reach the equilibrium state and converged to a stable conformation after 10.5 ns. While in the case of FCR-Os, the RMSD increased up to 7.5 ns and then it converged to a stable conformation at the end of the simulation. The overall atomic fluctuation for both proteins generated the root mean square atomic fluctuation (RMSF) plots (Figs. 5a, 6a) and provided the B-factor structure of the protein that reflected the fluctuation of atoms about their average positions (Laskowski et al. 1993; Batra et al. 2017). The flexibility of atoms was categorized by different colors and identified by B-Factor analysis of proteins, which provided important information regarding different stable and unstable parts of protein. The structural domains of FCR-LM structure (Figure S4a) had fewer fluctuations while the domains of FCR-Os (Figure S5a) had more fluctuations. Moreover, the average stable structures for both proteins were generated and validated. The validation scores, i.e., Ramachandran plot statistics, PROSA Z score, ERRAT score, VERIFY 3D score and overall G-factor scores significantly improved, which indicate that both structure reached their stable conformations. In-depth Ramachandran plot statistics indicates that 99.8 and 98.6% of total amino acid are found in generally allowed regions for FCR-LM and FCR-Os, respectively (Fig. 4Sb, S5b). Finally, these results infer the sequence of FCR-LM and FCR-Os are modeled accurately and the stable structure of both proteins obtained through MD simulations.

Fig. 4 **a** Total energy of profile for the FCR-LM (Black) and FCR-Os (Red) with respect to time of simulation. **b** RMSD plot showing the fluctuation height (nm) versus time (ns) of simulation

Fig. 5 **a** Calculated RMSF plot for atoms of FCR-LM versus fluctuation height (nm). **b** The B-factor representation of the FCR-LM protein with highly (red), medium (green) and less flexible (blue) atoms during dynamics simulation

Conclusions

High nutritive value of minor millets makes them potential candidates for identification and mining of genes/alleles that govern nutritional traits, including micronutrients viz Fe, Zn and Vitamin A. Previous studies on cereal crops has personified the importance of number of genes for grain Fe and Zn homeostasis that involves uptake, transport and loading, but so far no reports are available on candidate genes governing these traits in minor millets. This fact makes worth the search for genes implicated in grain Fe and Zn concentration in minor millets. Making efforts in this direction through this work, an ortholog of rice *OsFRO2* gene has been identified in RLM-37 genotype of LM followed by its comprehensive characterization employing computational genomics. Cloning and characterization for this gene is under progress. The overall bioinformatics analysis revealed a good correspondence between the identified gene in LM and *OsFRO2* gene of rice in terms of sequence, structure and functional similarity, thereby showing that the said genes are orthologous to each other. Through this study we have generated baseline data for the discovery of metal homeostasis genes in millet crops. Further, experimental procedures based on analyzing expression of *ferric chelate reductase* under Fe

Fig. 6 **a** Calculated RMSF plot for atoms of FCR-Os versus fluctuation height (nm). **b** The B-factor representation of the FCR-Os protein with highly (red), medium (green) and less flexible (blue) atoms during dynamics simulation

sufficient and deficient conditions is required to thoroughly understand its association with grain Fe/Zn contents and to validate these findings. The valuable genes or their orthologs, thus identified will serve as new sources and targets for manipulation for improving grain micronutrient levels. This also has implications to develop crop plants with improved nutritional characteristics and better thriving capability under nutrient-deficient soils.

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Author contributions Execution of experiments and analysis: MD and SG; Experiments designing: MD, SG and GC; Wrote paper: MD, SG, AP, ARR, and GC.

Compliance with ethical standards

Conflict of interest The authors do not have any conflict of interest.

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