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Bioinformatics characterization of lipoxygenase gene from pearl millet associated with rancidity

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Abstract
Despite its high nutritional value (9-11 percent protein, 6% fat, micronutrients like iron and zinc), and high calorie content (361 kcal/100 gm), the pearl millet crop is not generally consumed owing to rancidity in the flour, even when kept for a short period of time (less than 30 days). Lipase, lipoxygenase (LOX), polyphenol oxidase, and peroxidase are four enzymes that produce enzymatic rancidity. Lipase degrades lipids to produce free fatty acids, which serve as a substrate for LOX, which catalyzes the reaction to produce hydroperoxides, and then leads to the formation of aldehydes, ketones, and polymers of hydroperoxide, all of which are responsible for the development of off odors and ultimately rancidity. Based on substrate selectivity and the generation of several forms of hydroperoxides, the LOX 2 isoform was chosen for this investigation to investigate its effectiveness in preventing flour rancidity utilizing various treatments. The transcript produced from the transcriptome data was 1.411 kb long and included 921 amino acids, according to bioinformatics analysis. The cytoplasmically confined LOX 2 protein has 45 alpha helices, 74 beta turns, and 5 domains as identified using PDBsum. According on Cys REC analysis, the most likely pair to form a disulfide bond is located between positions 5-151, 202-915, and 209-299. The catalytic residue in LOX 2 was anticipated to be Asn^{720}, whereas amino acid residues His^{525} His^{530}, His^{716}, Asn^{720}, and Ile^{864} are involved in the formation of coordinating bonds with iron atoms. The amino acid residues His^{582}, His^{587}, His^{774}, and Asn^{778} are implicated in the ligand binding site, according to the 3D ligand binding tool.

Keywords: Lipoxygenase, rancidity, transcriptome

Introduction
By 2050, the world's population is expected to reach 9.1 billion people (FAO 2009) and the occurrence of severe climatic conditions is expected to rise from 1-6 °C by 2100. (The National Academies Press, 2010). Crops that are well suited to harsh climatic conditions and produce larger yields are the only way to attain food security. According to assessments from the Intergovernmental Panel on Climate Change (IPCC), it also impacts the availability of water and nutrients, which has a negative impact on crop productivity. Millets, particularly pearl millet, are an excellent choice for combating the aforementioned ailment.

Pearl millet (*Pennisetum glaucum* L.) is a cross-pollinated C4 cereal crop in the Poaceae family with a short life cycle and 1.79 GB genome (Varshney *et al*., 2017, Nature Biotechnology) [6]. Pearl millet protein content ranges from 7.3 percent to 13.86 percent, with a more balanced amino acid profile, and is high in vitamins (Vit A, Vit B1, Vit B2, Vit B3, folic acid and so on). Fat content ranges from 4.36 percent to 7.11 percent lipid (Anonymous 2011), with better fat digestibility than other cereals. Muthamilarasan *et al*. (2016) [3] found that pearl millet has a higher content of unsaturated fatty acid, accounting for 75% of total fat, and a relative abundance of nutritionally important omega-3-fatty acids such as oleic acid, linoleic acid, and linolenic acid, as well as a good source of saturated fatty acid. It is also an excellent source of antioxidants.

Despite its high nutritional value, it is less popular in society because of its short shelf life. The main cause of its shorter shelf life is lipid oxidation (rancidity), which causes an off-odor to develop during storage. Lipoxygenase (EC 1.13.1.12; linoleate: oxygen oxidoreductase) is an enzyme involved in the oxidative breakdown of lipids. It's a non-heme, iron-containing dioxygenase that catalyzes the conversion of polyunsaturated fatty acids and esters to hydroperoxides (Rodrigo *et al*., 2006) [4]. The oxidation of unsaturated fatty acids by LOX causes food quality to deteriorate (Hayward *et al*., 2017) [2] (1) loss of nutritional quality due to the destruction of the essential fatty acids linoleic, linolenic and arachidonic acids as a direct

result of the reaction and indirect degradation of vitamins and proteins by hydroperoxides and free radical intermediates; (2) further degradation of the hydroperoxides leads to the formation of volatile compounds such as aldehydes, ketones, and alcohols, which results in the development of an off-flavors that is often described as hay (Whitaker *et al*., 1996) [8]. Based on the rancidity problem in pearl millet and the fact that LOX is the principal cause of oxidation, the current research aims to better understand LOX activity for extending the shelf life of pearl millet.

Material and Methods

The nucleotide sequences of closely related cereals and legumes were obtained from the NCBI GenBank database, and the sequence similarity was determined using the NCBI BlastN (https://www.ncbi.nlm.nih.gov/) tool. The CELLO tool was used to investigate the protein's subcellular distribution. The physiochemical properties of the selected LOX sequences were examined using ProtParam software (http://web.expasy.org/cgi-bin/protparam/protparam). MEGA 7.0 (Molecular Evolutionary Genetic Analysis); (http://megasoftware.net/) software was used to create a phylogenetic tree to explore evolutionary relatedness using the neighbor joining method. ClustlW was used to run multiple alignments on the amino acid sequences to identify and shade the conserved amino acid sequences. MEME (multiple Em for motif elicitation) was used to find the conserved region and novel motifs within the LOX protein, which is hosted at the National Biomedical Computation Resource (http://meme.nbcr.net). MEME software (http://meme-suite.org) was used to predict conserved domains in the LOX protein sequences. The secondary structure prediction was done by using PDBsum (http://www.ebi.ac.uk/pdbsum). Homology based modeling of the LOX protein was accomplished using Phyre 2 software with c1no3A from *Glycine max* as the template. The predicted 3D structure of LOX protein was further validated by Ramachandran plot analysis using RAMPAGE program (http://mord red.bioc.cam.ac.uk/~rapper/rampage.php).

Results

The transcript obtained from the transcriptome data was 1.411 kb length. The coding sequence for LOX 2 was retrieved by using the BlastP tool of NCBI (Figure. 1). It showed a maximum homology sequence with Settaria it.

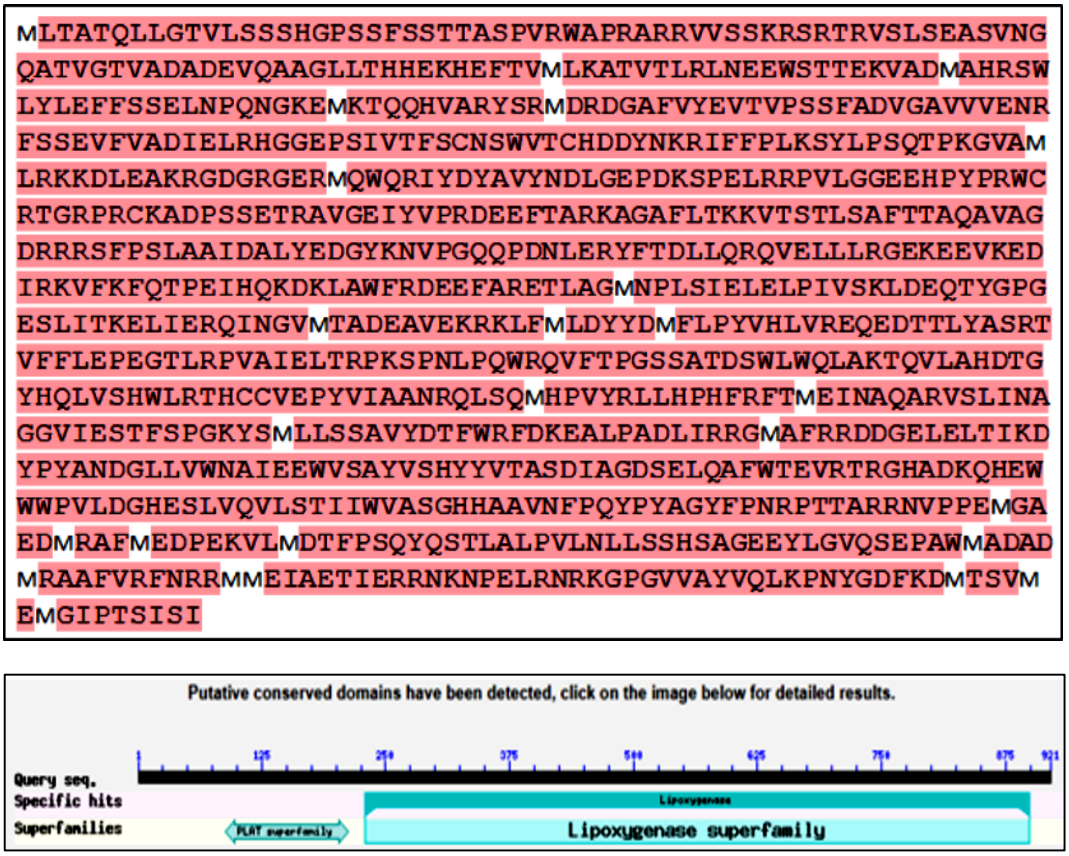


Fig 1: Pictorial representation of LOX 2 amino acid sequence (2766bp) retrieved from transcriptomic data of pearl millet and its putative conserved domain of LOX 2 enzyme

The Protparam analyses of all LOX proteins from various plant species included molecular weight (MW), theoretical pI, instability index, aliphatic index and hydropathicity index which revealed that the proteins have varying numbers and types of amino acids. Molecular weight of LOX protein from different plant sources varying in ranges from 84 kDa to 105 kDa. Isoelectric point varied from 6.5 to 8.6 with LOX 2 protein from pearl millet showing pI value of 8.6. Difference in the pI of LOX proteins from different plant species reflects variation in the signal peptide sequences at the N-terminal regions and it also helpful in studying the cellular localization of the enzymes. Instability index measure the in-vivo half-life of proteins. Analysis of LOX protein across crop species revealed that its instability index ranges from 35.61 to 52.31 with the estimated half-life is 30 hours. A protein whose instability index is <40 is generally considered to be stable whereas a value >40 predicts it to be of unstable in nature (Vinutha *et al*., 2015; Sahay *et al*., 2010; Guruprasad *et al*.,

1990) [7, 5, 1] (Table 1). Aliphatic index of selected crop ranges from 79-89 indicating its stable nature over wide range of temperature. Aliphatic index of pearl millet LOX 2 is 75.04. GRAVY (Grand Average of Hydropathy) is used to determine hydrophobicity of the whole protein. Its value lies in between +2 to -2. Gravy indices of LOX 2 protein found to be -0.336 indicating that its molecular surface contains more hydrophilic amino acid suggesting that molecular surface of LOX 2 protein has higher hydrophilicity indicating its greater interaction with water (Table 1). ClustalW is used for aligning multiple nucleotide or protein sequences in an efficient manner. Sequence alignment of LOX protein from selected crops was done using ClustalW and it was found that "YNDLGNPDR", "LIEDYPYAVDGL", "AAVNFGQYPYGGF" amino acid stretch are some of the conserved regions in LOX from selected crop species.

Table 1: Comparison of Physico chemical properties of LOX enzyme from pearl millet with the related LOX from different monocot and dicot plants using ProtParam tool

Crop	Number of amino acid	Mol. wt	pI	No of -ve amino acid	No of +ve amino acid	E.C. (premolar/cm)	Instability index	Aliphatic index	Gravy
Pearl millet LOX 2	921	104979.2	8.6	122	108	162525	47.4	75.04	-0.336
Hordeum vulgare	913	101164.14	6.41	111	102	123355	44.12	85.95	-0.307
Aegilops tauschii	922	102452.52	6.71	112	107	123355	47.96	84.15	-0.350
Sorghum bicolor	924	102928.43	8.60	106	112	122730	47.98	83.79	-0.365
Setaria italica	917	102043.56	7.43	108	108	123355	43.83	85.07	-0.329
Panicum hallii	922	102847.38	7.71	108	109	128855	47.62	84.50	-0.357
Zea mays	922	103039.62	8.60	108	114	123230	49.44	83.95	-0.378
Oryza sativa Japonica	918	101959.30	6.95	109	106	130220	45.90	86.55	-0.318
Brachypodium distachyon	915	102258.35	6.77	119	116	120835	52.31	79.16	-0.403
Cajanus cajan	756	85339.24	6.49	90	84	127910	35.61	88.48	-0.336

The phylogenetic tree for the LOX protein revealed three distinct groups. The first group includes LOX isoforms from various monocot crops, whereas the second group includes monocot crops such as pearl millet and Settaria italica. There are LOX 2 isoforms present, as well as LOX from a dicot crop, *Cajanus cajana. Setarria italica* has a stronger resemblance to LOX 2 from pearl millet.

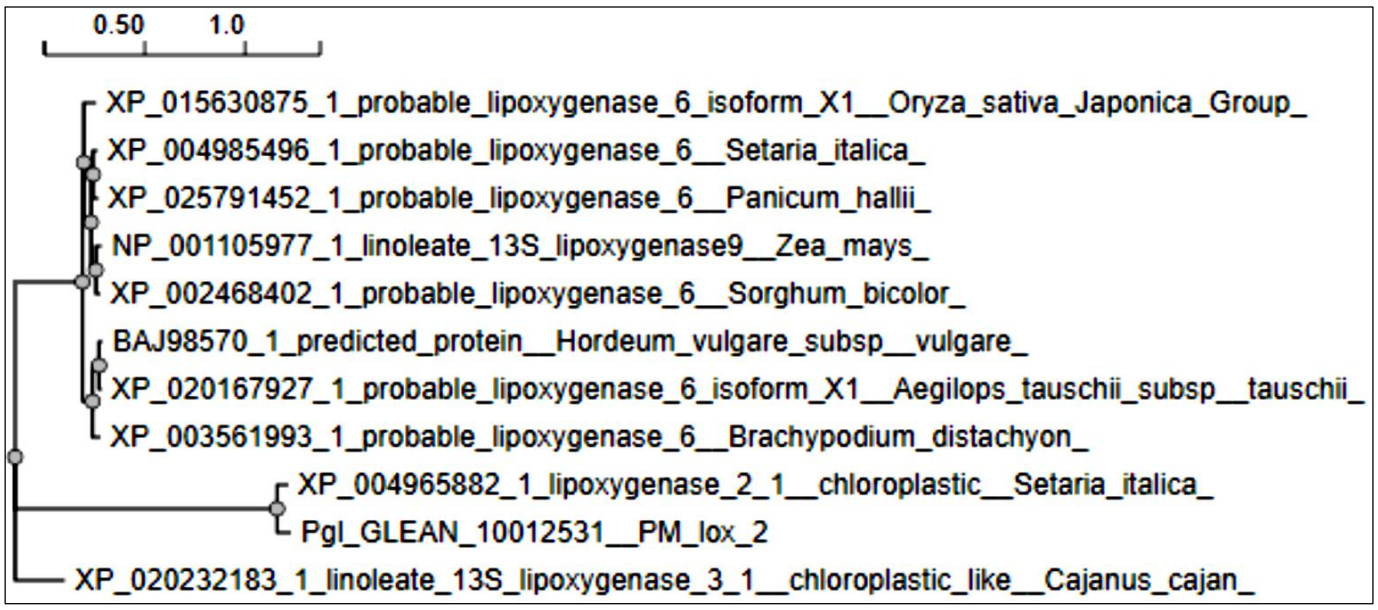


Fig 2: Phylogeny of LOX 2 proteins from selected plant species showing average distance among different LOX protein

ClustalW is used for aligning multiple nucleotide or protein sequences in an efficient manner. Sequence alignment of LOX protein from selected crops was done using ClustalW and it was found that "YNDLGNPDR", "LIEDYPYAVDGL", "AAVNFGQYPYGGF" amino acid stretch are some of the conserved regions in LOX from selected crop species.

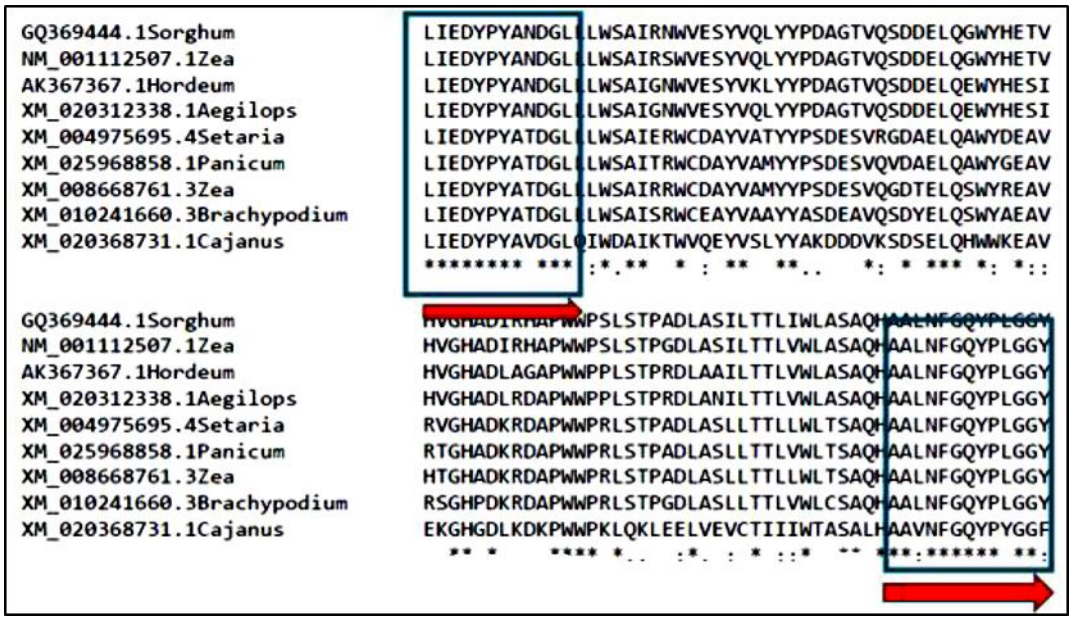


Fig 3: Amino acid sequence alignment of LOX protein from different crops using ClustalW and the blue boxes representing the conserved region of LOX protein

MEME analysis software was used to anticipate the motif. The research indicated the presence of three highly conserved motifs, namely motif 1, motif 2 and motif 3, in all of the LOX proteins used in the experiments. The separation of these three motifs was found to be similar, indicating structural conservation. Multiple sequence alignment revealed that motif 1 is located between 360 and 450, motif 2 between 550 and 620 and motif 3 between 625 and 680. None of these motifs, however, were annotated for their functional significance.

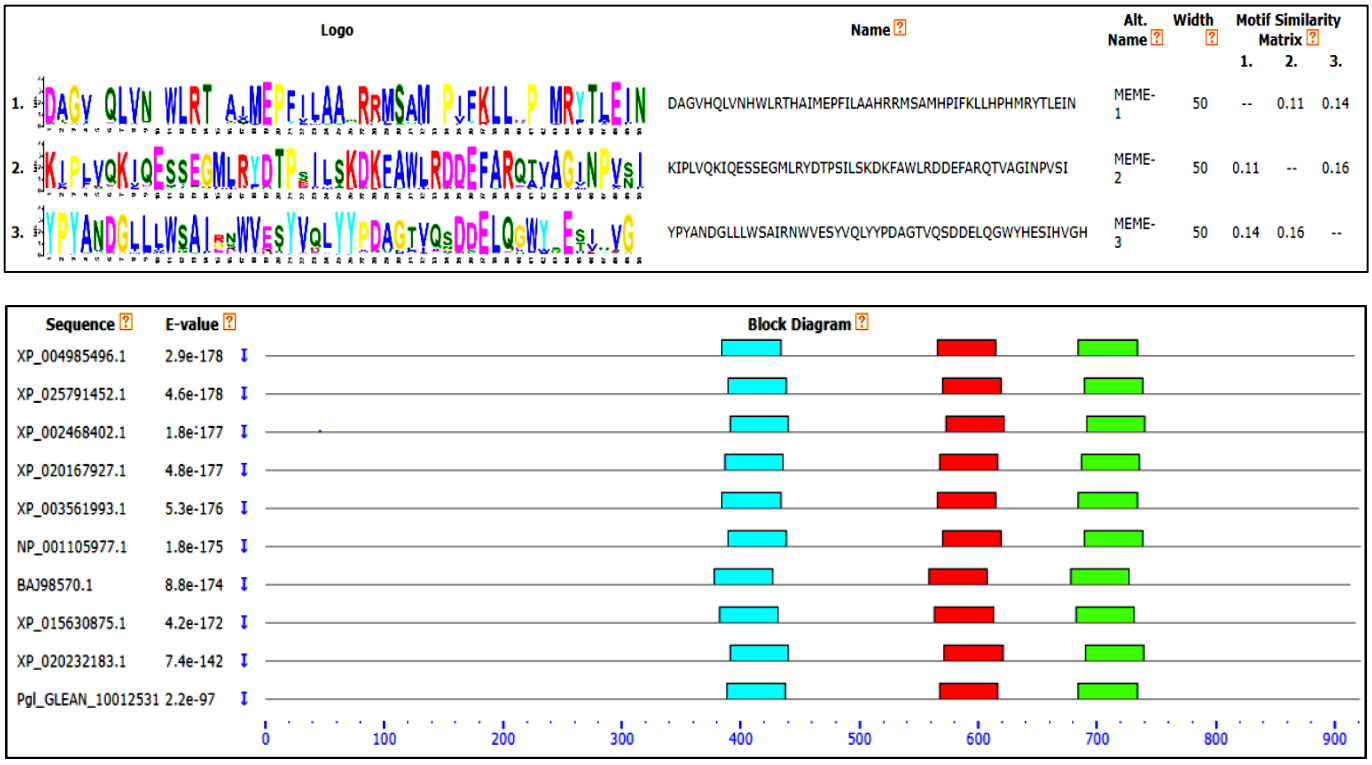
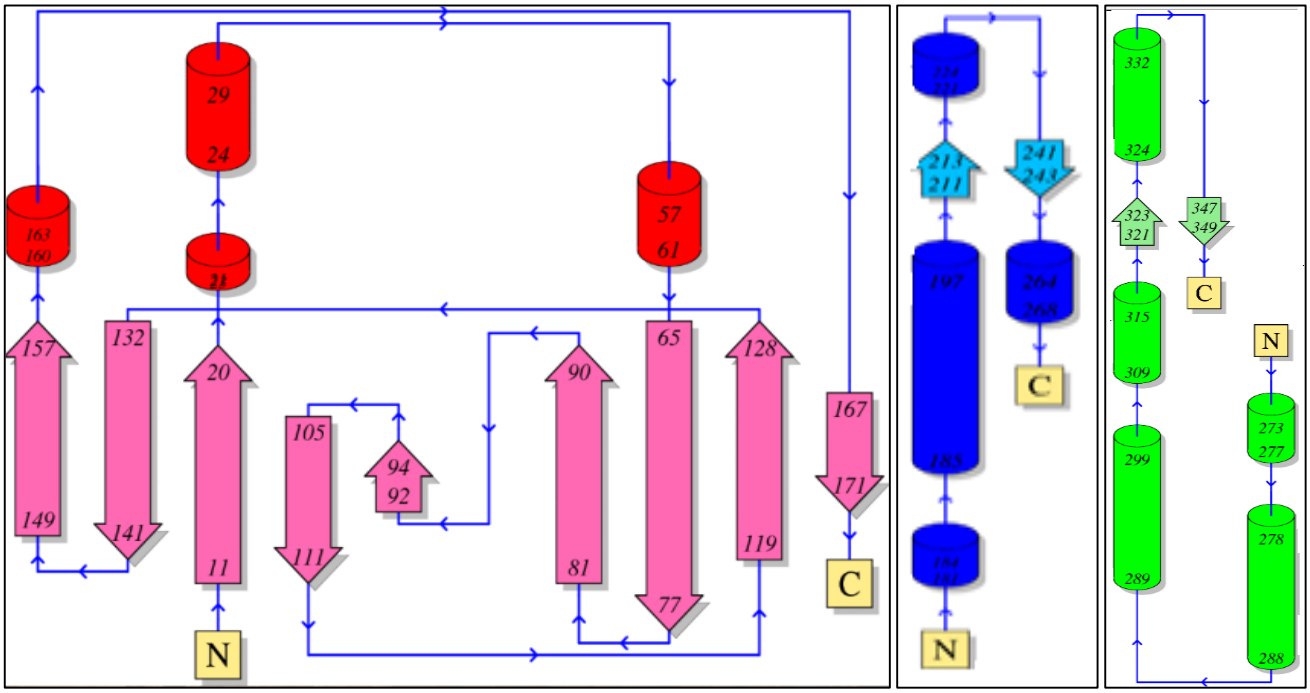


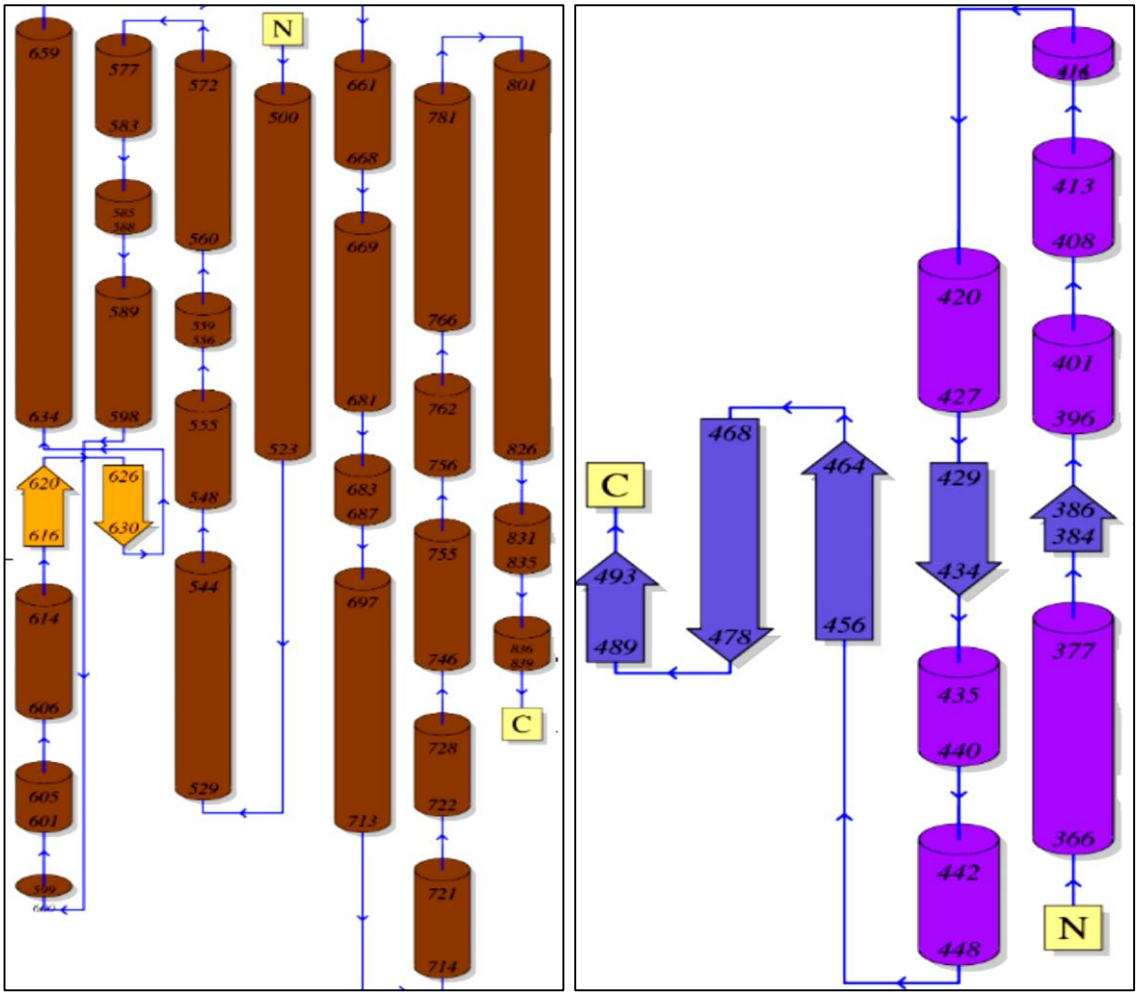
Fig 4: Motif analysis by MEME; (A). Amino acid sequence logos of motif 1, motif 2 and motif 3; (B). Distribution of conserved motifs in LOX proteins as identified by MEME. Different motifs are indicated with different color boxes

PDBsum software is used to determine the secondary structure and disorders present in the protein. Secondary structure prediction of pearl millet LOX 2 revealed 37% amino acid residues are present in alpha helix while 11% of amino acid is present in beta strand. 27% disorder structure present in LOX 2. The greater occurrence of alpha helices (37%) in LOX 2 proteins revealed its stable nature based on the hydrogen bonding nature of the α-helices which acts as one of the main forces of secondary structure stabilization in proteins. The topology of LOX 2 protein from pearl millet showed that, it consists of five structural domains. Domain 1 has predominance of beta strand while Domain 5 mainly consists of alpha helices. Secondary structure and domain analysis was done using PDBsum and found that it comprises

of 45 alpha helix, 74 beta turn, 10 beta hairpins and 62 helix-helix interacs. It was also predicted that in LOX 2 the catalytic residue was found at Asn[720] while amino acid residues His[525] His[530], His[716], Asn720 and Ile[864]are involved in coordinate bond formation with iron atom.



Domain 1 **Domain 2** **Domain 3**



Domain 4 **Domain 5**

Fig 5: Pictorial representation of secondary structure and disorder prediction of LOX 2 protein using PDBsum software

To envisage the homology model of LOX 2 from pearl millet, researchers used the Phyre 2 software and the PDB template c1no3A from Glycine max. With 100 percent confidence, 809 residues (about 88 percent of the sequence) have been predicted. Ramachandran plot using RAMPAGE was used to further validate the predicted 3D model from Phyre 2. The plot demonstrated that 89 percent of amino acid residues were found in the most favored locations, while only 6.5 percent were dispersed throughout the generally permitted range. As a result, the predicted LOX 2 model by the Phyre 2 programme was found to be a good model. The Cys REC analysis software was used to predict the secondary structure, and it was discovered that there was a total of 9 cystein residues at position 5, 52, 151, 202, 209, 299, 639, 895, 915. Between positions 5-151, 202-915 and 209-299, the most likely pair to form a disulfide bond was discovered.

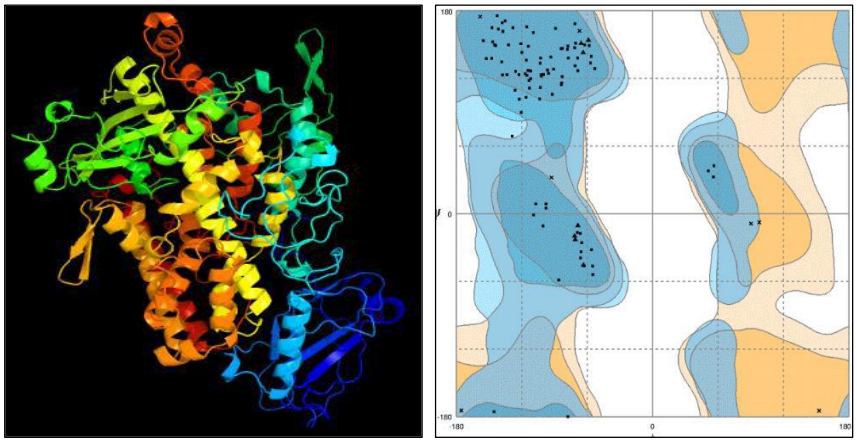


Fig 6: Predicted 3D structure of the pearl millet LOX 2. The model was generated with PDBsum using PDB templatec1no3A to visualize the model. (B) The Ramachandran plot for the modeled LOX 2 of pearl millet generated by using RAMPAGE

We can utilize CELLO software to determine where LOX is located within the cell. According to the CELLO results, LOX is primarily found in the cytoplasm, with some isoforms also found in other cellular compartments such as the inner membrane, periplasm and outer membrane, as well as a small amount in the extracellular matrix.

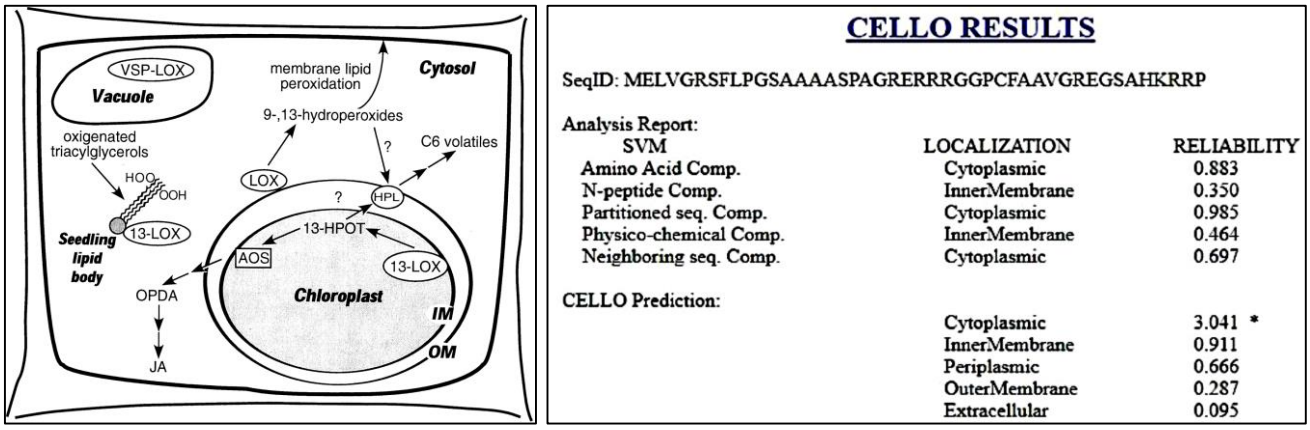


Fig 7: Prediction of Cellular localization of LOX 2 protein using Cello software

The ligand binding site of the LOX protein was determined using a ligand binding site prediction tool called 3D ligand binding site, indicating that this ligand binding site may be the catalytic binding site for unsaturated fatty acids with methylene carbon at C9 or C11 position and O2, as these two serve as potential substrate for LOX. The amino acid residues His^{582}, His^{587}, His^{774}, and Asn^{778} were found to be involved in the ligand binding site using 3D ligand binding techniques.

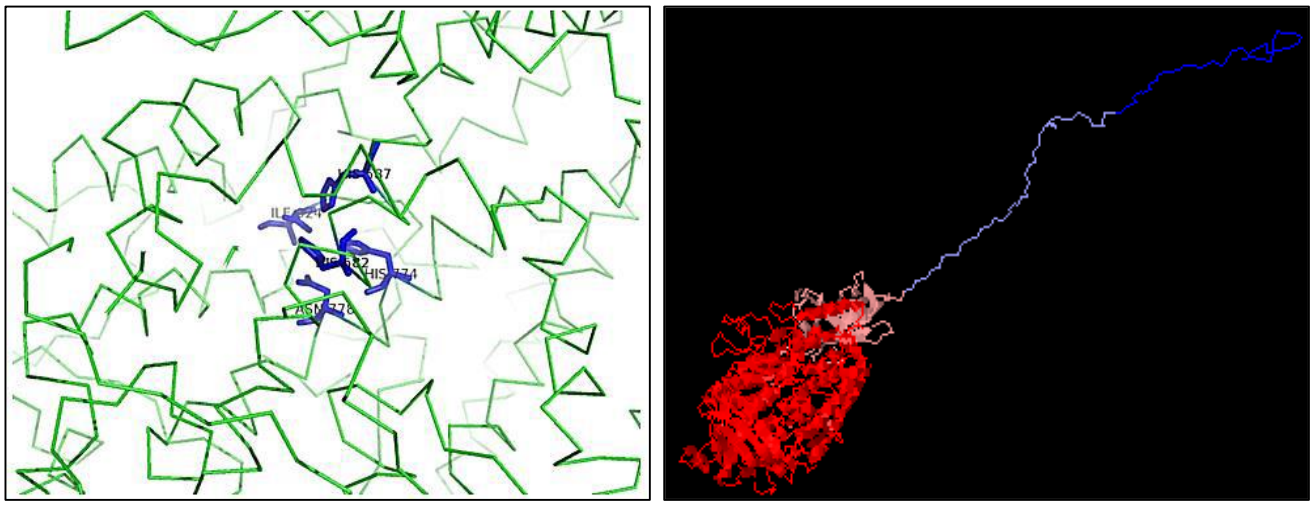


Fig 8: Prediction of ligand binding site in LOX 2 protein of pearl millet by using 3D ligand binding site tool. Blue color region indicating the ligand binding site

STRING software was used to analyze protein-protein interactions, which revealed connections with LPAT (Lysophosphatidic acid acyltransferase), PDAT (Phospholipid: diacylglycerol acyltransferase), Lactoyl glutathione lyase, and the Cyt P450 family.

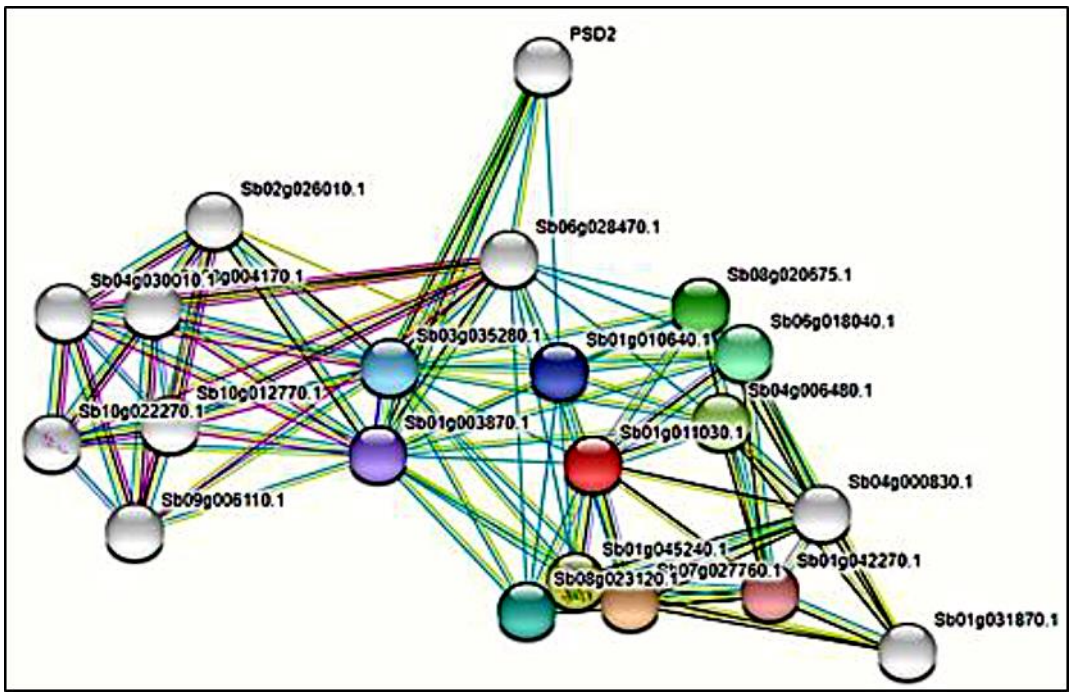


Fig 9: Prediction of ligand binding site in LOX 2 protein of pearl millet by using 3D ligand binding site tool. Blue color region indicating the ligand binding site

Conclusion

Despite its high nutritional value (9-11 percent protein, 6% fat, micronutrients like iron and zinc), and high calorie content (361 kcal/100 gm), the pearl millet crop is not generally consumed due to rancidity in the flour, even when stored for a short period of time (less than 30 days). Lipase, lipoxygenase (LOX), polyphenol oxidase, and peroxidase are four enzymes that produce enzymatic rancidity. Lipoxygenase (EC 1.13.11.12) is a dioxygenase that contains iron. The oxidation of polyunsaturated fatty acids and esters into their corresponding hydroperoxides is catalyzed by LOX. In plant tissue, there are several LOX isoenzymes with different pH optimums, isoelectric points, and other features. LOX is also involved in the synthesis of volatile taste molecules in fruits and vegetables that give them their "fresh" and "green" qualities.

The transcriptome data yielded a transcript with a length of 1.411 kb. Using NCBI's BlastP programme, the coding sequence for LOX 2 was discovered. It shared the highest degree of homology with Settaria italica. Protparam is made up of 921 amino acids with a 47.4 instability index, according to research. Its hydrophilic character was shown by the negative value of GRAVY indices. PDBsum was used to analyze the secondary structure and domains, and it was discovered that it has 45 alpha helixes, 74 beta turns, and 5 domains. Using CELLO software, the cytoplasmic localization of this protein has been predicted. The constructed 3-D homology model was confirmed, and the Ramachandran plot revealed 90% of amino acids in preferred areas. At both the N-terminal and C-terminal sections, which

contain proline and other bulky amino acids, the structure was discovered to contain a few disordered amino acids. According on Cys REC analysis, the most likely pair to form a disulfide bond is located between positions 5-151, 202-915, and 209-299.

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