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RESEARCH

Genome-Wide Identification and Expression Analysis of *Lipoxygenase (LOX)* Gene Family in Pearl Millet (*Pennisetum Glaucum L.*)

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Abstract

Lipoxygenases (LOX) are non-heme, non-sulfur iron-containing enzymes involved in lipid oxidation, playing key roles in plant development and stress responses. Despite their functional significance, *LOX* genes remain poorly characterized in climate-resilient crops like pearl millet (*Pennisetum glaucum*), which is prone to grain rancidity—a major post-harvest problem that affects quality and shelf life. In this study, ten *PgLOX* genes were identified and classified into 9-LOX and 13-LOX subfamilies, with uneven distribution across chromosomes and evidence of tandem and segmental duplications. Evolutionary analysis indicated purifying selection. Motif analysis revealed conserved motifs within the *PgLOX* gene family. *Cis*-motif analysis showed that the potential promoter regions of the *PgLOX* genes contain various elements that are tissue-specific, related to development, stress-responsive, and hormone-responsive. Protein–protein interaction predictions suggested functional connections among eight PgLOX proteins. qRT-PCR analysis showed that among the ten *PgLOX* genes, *PgLOX1* was significantly expressed in the grains of genotypes susceptible to rancidity, whereas it was not present in leaf tissues. These findings indicate that *PgLOX1* may be involved in lipid peroxidation and oxidative rancidity in pearl millet grains, emphasizing its potential for functional validation and gene editing to enhance grain quality.

Main Conclusion The *Pennisetum glaucum L.* gene likely contributes to oxidative rancidity in pearl millet grains and may be a potential candidate for gene editing to improve grain quality and extend shelf life.

Keywords Gene expression · Lipoxygenases · Pearl millet · Off-flavor · Rancidity · Grain quality

Introduction

Among all millets, pearl millet [*Pennisetum glaucum* (L.) R. Br.] is the most widely grown and the sixth most important hardy dryland cereal grain crop with a genome size of ~ 1.79 Gb (Varshney et al. 2017). It is cultivated on approximately 27 million hectares worldwide, mainly in arid and semi-arid regions of Asia and Sub-Saharan Africa (SSA). Pearl millet is a resilient and nutritious crop frequently grown by smallholder farmers and known as the "poor man's wheat" in India (Goswami et al. 2020). Millets have significant potential to grow well under harsh environmental conditions (Tiwari et al. 2022). With these remarkable attributes, pearl millet stands out among key crops like rice, wheat, and maize. Recognizing its significance, the United Nations (UN) declared 2023 as the "International Year of Millets."

Pearl millet is a highly nutritious and climate-resilient crop; however, its consumption remains limited due to the poor shelf life of its flour. A major challenge is the rapid onset of rancidity after milling, which produces an unpleasant odor and off-flavor, making the flour unpalatable (Tiwari et al. 2022; Aher et al. 2022). This leads to significant food waste and increases the workload for women who often grind fresh grains daily. Rancidity in pearl millet is a multifactorial problem influenced by changes in lipid composition (Goswami et al. 2019), metabolites (Yogendra et al. 2024),

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and the presence of volatile compounds (Aher et al. 2022). Additionally, oxidative degradation of unsaturated fatty acids (Bhargavi et al. 2024) and increased peroxidase activity (Goyal and Chugh 2014) worsen the problem. Although post-milling strategies have been explored to reduce rancidity, these methods are often labor-intensive, expensive, difficult to implement on a larger scale, and may compromise nutritional quality (Aher et al. 2022; Goyal et al. 2015).

Intracellular enzymes, such as lipases, lipoxygenases (LOXs), peroxidases (POXs), and polyphenol oxidases (PPOs), along with factors like light, moisture, temperature, and metal ions, contribute significantly to the deterioration of the flour's taste and aroma (Kumar et al. 2021). Enzymes play a crucial role in the lipid hydrolysis and oxidation of fatty acids (Rani et al. 2018). Lipases mainly participate in hydrolytic rancidity by hydrolyzing long-chain polyunsaturated fatty acids (PUFAs) into diacylglycerols (DAG), monoacylglycerols (MAG), and free fatty acids (FFA) (Sravani et al. 2023). Our previous study showed that triacylglycerol (TAG) lipases contribute to hydrolytic rancidity in milled flour after 5 to 7 days of storage (Aher et al. 2022). LOXs and POXs further oxidize these PUFAs and FFA into hydroperoxides and secondary carbonyl compounds like aldehydes and ketones. Pearl millet is rich in PUFAs such as oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3), which are the main targets for rancidity-causing enzymes like LOXs.

In plants, LOXs are crucial for lipid metabolism and act as signalling molecules that influence various cellular activities. These activities include seed germination, responses to both biotic and abiotic stresses, fruit ripening, wound healing, cell death, senescence, and the production of stress hormones (Viswanath et al. 2020; Singh et al. 2022). *LOX* genes are categorized into subclasses based on their regio-specificity, size, and interactions with other proteins. These subclasses include 5-LOX, 8-LOX, 9-LOX, 10-LOX, 12-LOX, 13-LOX, and 15-LOX, collectively known as classical LOXs. In plants, the primary enzymes are 9- and 13-LOXs (Viswanath et al. 2020; Singh et al. 2022), while in animals, 5-, 12-, and 15-LOXs play essential roles (Upadhyay et al. 2019; Erba et al. 2024). Genes encoding *LOX* have been identified across various plant species at the genome-wide level. These include grapevine (18) (Podolyan et al. 2010), Arabidopsis (6), rice (14) (Umate 2011), maize (13) (Ogunola et al. 2017), red pepper (8) (Sarde et al. 2018), sorghum (9) (Shrestha et al. 2021), foxtail millet (13) (Zhang et al. 2021), and wheat (44) (Wang et al. 2023), potato (17) (Zhu et al. 2024). Different members of LOX serve various roles in plants. For example, *OsLOX2* contributes to seed longevity in rice (Huang et al. 2014), *AtLOX1* is involved in defence response in *Arabidopsis* (Melan et al. 1993), *DkLOX3* shows resistance to drought and high salinity while upregulating stress-responsive genes in persimmon (Hou et al. 2015), and *CaLOX1* from pepper enhances drought and salt tolerance in the transgenic *Arabidopsis* (Lim et al. 2015).

Besides their typical functions, LOXs can produce undesirable aromas and off-flavors, and they can decrease flavor stability. *LOX* genes cause oxidative rancidity in rice (RoyChowdhury et al. 2016; Sinha et al. 2020). LOX enzymes are found in the bran part of rice grains. After milling, these enzymes react with stored lipids/triacylglycerols (TAG), causing oxidation that results in off-flavors and odor (Sinha et al. 2020). *OsLOX3* and *OsLOX14* are mainly expressed in the bran of germinating rice grains, where they may contribute to the rancidity of rice bran oil (Sinha et al. 2020). The expression levels of two *LOX* genes, *9-LOX1* and *L-2*, were significantly higher in active bran and seed tissues. *R9-LOX1* is involved in synthesizing nonanal, acetic acid, and hexanal in rice bran or seed tissues (RoyChowdhury et al. 2016). Moreover, studies in rice and soybeans have shown that the absence of *LOX* genes or *LOX* mutants improves storage quality (RoyChowdhury et al. 2016) and reduces the beany flavors (Wang et al. 2020). A mutation in the barley *lipoxygenase 1* (*lox-1*) gene significantly decreases the production of 9-hydroperoxy-octadecanoic acid, which in turn lessens off-flavors in brewed products during storage (Hirota et al. 2006). Additionally, knocking down *TaLpx* genes in wheat using RNA interference (RNAi) significantly enhances flour processing quality by altering levels of unsaturated fatty acids and other starch components (Lv et al. 2021).

Despite its remarkable climate resilience and high nutritional value, pearl millet has limited global acceptance, mainly because of its flour's short shelf life. Understanding the mechanism of rancidity and the role of intracellular enzymes such as lipoxygenases (LOXs) in lipid oxidation and other cellular processes is essential. Few efforts have been made to explore the role of *LOX* genes in pearl millet. Chitranashi et al. (2024) studied the characterization of the *PgLOX2* gene sequence in pearl millet using in silico methods. Sharma and Chugh (2017) purified and characterized two isoforms of LOX from pearl millet grains. Apart from this, comprehensive studies are still needed to identify and characterize *LOX* genes in pearl millet. This study comprehensively analyzes the *LOX* gene family at the whole-genome level, using data from the pearl millet genome and various bioinformatics tools. We identified ten members of the *PgLOX* gene family in the pearl millet genome and analyzed their chromosomal locations, gene structures, subcellular localizations, protein interaction networks, *cis*-motif analyses, and phylogenetic relationships of the encoded proteins. Additionally, we examined the expression patterns of *PgLOXs* in the pearl millet grains from two pearl millet genotypes with different levels of rancidity using quantitative real-time PCR (qRT-PCR). Our results provide a basis

for further research on understanding the role of the pearl millet *LOX* gene family in grain and flour quality (Supplementary Fig. 1).

Materials and Methods

Identification of *PgLOX* Genes in Pearl Millet

To identify the *LOX* gene family in pearl millet, we used LOX protein sequences from rice (Umate 2011), sorghum (Shrestha et al. 2021), and foxtail millet (Zhang et al. 2022) as queries in a BLAST search on Milletdb (http://milletdb.novogene.com/) (Sun et al. 2023). The genome sequence information of the cultivated genotype (PI583800) was used as a reference to extract the *PgLOX* genes. A comprehensive Hidden Markov Model (HMM) search was performed using TBtools to validate the identified protein sequences and ensure accuracy (Chen et al. 2020). Pfam identifiers for the PLAT/LH2 (PF01477) (polycystin-1, lipoxygenase, -toxin domain, or lipoxygenase homology) and Lipoxygenase (LOX) (PF00305) domains were used to access an HMM library obtained from the Pfam database (https://www.ebi.ac.uk/interpro/download/Pfam/). After removing redundant sequences, we analyzed the remaining sequences for PLAT and LOX domains using the InterProScan tool (https://www.ebi.ac.uk/interpro/search/sequence/). Sequences containing only the Lipoxygenase (LOX) or PLAT domains were excluded. The protein's molecular weight (m.wt), isoelectric point (pI), number of amino acids, aliphatic index, and GRAVY were predicted using Expasy ProtParam (https://web.expasy.org/protparam/). Gene structures of pearl millet LOXs were mapped using GSDS (https://gsds.gao-lab.org/) software. The sub-cellular localization of *PgLOX* genes was predicted using the DeepLoc tool in TBtools (https://services.healthtech.dtu.dk/services/DeepLoc-1.0/). Additionally, gene mapping, which included the relative distances on the chromosome, was performed using TBtools (Chen et al. 2023).

Phylogenetic, Motif Analysis, and Protein–Protein Interactions of PgLOXs

The amino acid sequences of the *LOX* gene family members from pearl millet, rice, sorghum, and foxtail millet were aligned using MEGA11 software with default parameters. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA11) with the neighbor-joining (NJ) method, with the Poisson substitution model, and 1000 bootstrap replicates under standard settings.

The conserved motifs of the PgLOX proteins were identified using the Multiple Em for Motif Elicitation (MEME) (https://meme-suite.org/meme/tools/meme) with Zero Or One Occurrence per Sequence (zoops) site distribution, and the maximum number of motifs was limited to 8, with lengths varying from 10 to 50 amino acids. The obtained results were visualized using TBtools. To predict the potential protein-interaction network, the identified PgLOX proteins were submitted to STRING v11 (http://string-db.org) (Szklarczyk et al. 2019). The interaction confidence threshold was set at 0.7, and proteins predicted not to interact were excluded from the analysis.

Cis-Element Analysis in the Promoter of *PgLOX* Genes

To identify potential *cis*-acting elements, we extracted 1000 base pairs of genomic DNA sequences upstream of the initiation codon (ATG) of the *PgLOX* genes from Milletdb (http://milletdb.novogene.com/home). We then used the plantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify *cis*-acting elements in the promoter regions (Lescot et al. 2002). Finally, we visualized the identified *cis*-acting elements with TBtools (Chen et al. 3019).

Synteny, Ka/Ks, and Divergence Time Analysis of *PgLOX* Genes

Synteny analysis was performed with the Multiple Collinearity Scan toolkit (McScanX) in TBtools (Wang et al. 2012). The study used Blastp with the parameters set to CPU BlastP-2, E-value of $1e^{-10}$, and a maximum of 5 Blast-Hits per query. This method allows gene duplication analysis in pearl millet, rice, sorghum, and foxtail millet. The genome and annotation files for these crops were prepared and used as input for the One Step MCScanX Wrapper function. Next, the Multiple Synteny Plot function in TBtools creates a synteny diagram.

To assess the role of gene duplication in expanding the LOX gene family in pearl millet, we identified paralogous genes by constructing a phylogenetic tree with at least ≥ 70% alignment coverage (Gu et al. 2002). Phylogenetic analysis identified orthologous gene pairs among pearl millet, rice, sorghum, and foxtail millet. Later, TBtools were used to calculate the Ka/Ks ratios and explore the selective pressure acting on duplicated genes (Chen et al. 2023). The divergence time between *LOX* gene pairs was calculated using the formula $T = Ks/(2\lambda \times 10–6)$, where λ is 6.5×10^−9, denoting synonymous sites per year (Gaut et al. 1996).

Tertiary Structure Prediction of PgLOX Proteins

The tertiary structures of PgLOX proteins were predicted using a homology-based method with Swiss Model (https://swissmodel.expasy.org). We searched the SWISS-MODEL

template library for templates to predict tertiary structures using BLAST, HHblits, and AFDB. We evaluated the quality of the models using QMEAN scores.

Plant Materials and Treatments

Seeds from two pearl millet genotypes, which differ in rancidity (ICMB95222: low rancid and ICMB863: high rancid) (Aher et al. 2022), were obtained from the pearl millet crop breeding group of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. A portion of dried pearl millet seeds from two contrasting genotypes (ICMB95222 and ICMB863) was ground into a fine powder and used for RNA isolation. Additionally, some seeds were sown in the soil in the glasshouse under a 16-h light and 8-h dark photoperiod at a temperature of 30 ± 2 °C with a relative humidity of 70 ± 5% to collect leaf samples. Three biological tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was isolated using a Nucleospin RNA Plant kit (Macherey-Nagel, Germany) following the manufacturers' guidelines, with slight modifications: RA1 buffer was used for leaf tissue, and RAP buffer was used for grain power. The quality and quantity of the isolated RNA were assessed using a 1.4% agarose gel and a Qubit-4 fluorometer (Thermo Fischer, USA), respectively. Two micrograms of total RNA were reverse transcribed into first-strand cDNA using a SuperScript III first-strand cDNA synthesis kit (Invitrogen, NY, USA) following the manufacturer's protocol. The qRT-PCR was conducted with 1 µl of diluted cDNA (diluted 1:5 from the original cDNA) on a CFX96 Real-Time System (Bio-Rad, Hercules, USA), using 2X SensiFAST TM SYBR No-ROX (Bioline, UK) according to the manufacturer's instructions. The primers for each *LOX* gene were designed with the Primer3web tool (Untergasser et al. 2002), producing amplicons that range from 90 to180 base pairs. The qRT-PCR reactions were carried out by following the standard thermal profile: 95 °C for 2 min and then 30 cycles at 30s at 95 °C, 25s at 62 °C annealing temperature and extension of 25s at 72°C. After the 30th cycle, amplicon dissociation curves were measured for each gene. Each qRT-PCR reaction included three biological and three technical replicates. The expression levels were analyzed by normalizing them to the reference gene *PgEIF4a* (Reddy et al. 2015). The statistical significance of the expression was analyzed using a one-way Analysis of Variance (ANOVA) and the Student's *t*-test, both performed with the Microsoft Excel Data Analysis Toolpak. Significant differences ($P < 0.05$) were observed between group comparisons, as indicated by asterisks in the bar diagrams. Non-significant differences ($P \geq 0.05$) were marked as "ns." The primer sequence details used for the qRT-PCR are in Supplementary Table 1.

Co-Expression Network Analysis and Functional Enrichment

Raw RNA sequencing count data from pearl millet panicles exposed to high temperatures (BioProject PRJNA926343) were obtained from Figshare (https://figshare.com/articles/figure/Height_and_seed_setting_rates_of_pearl_millet_cultivars_with_different_tolerance_to_a_high_temperature/24532792) and correspond to the dataset described by Lou et al. (2024). Gene identifiers were mapped to updated IDs using an external mapping file (*Pennisetum glaucum* accession PI583800, Milletdb, Novogene); duplicate IDs were combined by summing their counts. Variance-stabilizing transformation (VST) was applied using DESeq2 (v1.38.0) to normalize raw counts. Lowly expressed and low-variance genes were removed (row mean ≤ 1 or variance ≤ 0.1). The resulting matrix was used as input for WGCNA (v1.72-1). A soft-thresholding power was selected with pickSoftThreshold, and a signed bicorrelation network was created with blockwiseModules (corFnc = "bicor," maxPOutliers = 0.05, minModuleSize = 30, mergeCutHeight = 0.25). Modules were automatically identified through hierarchical clustering and dynamic tree cutting. Lipoxygenase 1 (PgLOX1) genes served as targets to identify co-expressed partners within their respective WGCNA modules. Gene–gene bicorrelations were calculated, and only strong correlations ($|r| \geq 0.6$) were retained. The resulting network was exported as an edge list and visualized in Cytoscape (v3.9.1). Functional enrichment analysis of PgLOX1-related co-expression modules was performed using MilletDB (http://milletdb.novogene.com/tools/enrichment) for Gene Ontology (GO) terms and KEGG pathways, with default settings and significance evaluated through *P*-values. The enriched GO terms and KEGG pathways were visualized as horizontal bar plots in R (v4.3.1) using the ggplot2 package.

Results

Identification of *LOX* Gene Family Members in Pearl Millet

A total of ten *PgLOX* genes were identified in the cultivated pearl millet genome (PI157800). The protein sequences of these ten LOXs were analyzed further using the ScanProsite tool and NCBI Batch CD-search databases to identify the conserved domains of PLAT/LH2 and LOX (Table 1). The sequences containing PLAT/LH2 and LOX domains were

Table 1 Identification and physicochemical properties of ten PgLOX family members. The characterization of PgLOX members includes the positions of identified domains, the total number of exons, the length of the coding sequence (CDS), the number of amino acids, molecular weight (M.W.), isoelectric point (pI), aliphatic index, GRAVY, and subcellular localization

Gene name	Gene ID	Chr	Genomic sequence (bp)	ORF (bp)	No. of exons	Protein properties							
						Number of amino acids	Molecular weight (KDa)	PLAT domain position	Lipoxygenase domain position	pI	Aliphatic index	GRAVY	Subcellular localization
PgLOX1	PMD1G00143.1	chr1	3853	2748	8	915	104.728	88–174	188–894	5.9	77.37	−0.544	Cytoplasm
PgLOX2a	PMD2G01012.1	chr2	3228	2697	4	898	101.329	135–202	216–881	5.8	82.95	−0.351	Plastid
PgLOX2b	PMD2G06675.1	chr2	4634	2595	7	864	96.253	51–158	172–841	5.6	87.94	−0.274	Cytoplasm
PgLOX2c	PMD2G06676.1	chr2	5652	2664	9	887	100.379	64–164	178–863	6	87.06	−0.399	Cytoplasm
PgLOX3	PMD3G02769.1	chr3	3892	2772	7	923	102.906	160–222	236–906	6.3	80.53	−0.406	Plastid
PgLOX4	PMD4G04092.1	chr4	7297	2889	5	962	105.232	170–250	276–945	6.2	79.58	−0.314	Plastid
PgLOX5a	PMD5G00722.1	chr5	6061	2733	9	910	101.273	149–212	226–893	7.2	85.62	−0.33	Plastid
PgLOX5b	PMD5G04728.1	chr5	3254	2772	5	923	105.31	151–212	229–901	6	75.22	−0.454	Plastid
PgLOX6a	PMD6G06509.1	chr6	4637	1443	7	480	54.149	59–152	166–445	9.5	77.65	−0.546	Cytoplasm
PgLOX6b	PMD6G06510.1	chr6	3052	2604	5	867	96.773	54–161	175–846	5.9	86.51	−0.285	Cytoplasm

considered *LOX* genes. The PgLOXs are named based on their chromosomal location. If multiple members are found on the same chromosome, we assign letters such as a, b, c, and so on after the corresponding chromosome number in gene nomenclature. Ten *PgLOX* genes were unevenly distributed across six of the seven chromosomes (Fig. 1). Chromosome (Chr) 2 has the maximum number of *PgLOX* genes, totalling three. Chromosome 5 has two *PgLOX* genes, while chromosomes 1, 3, and 4 each have one *PgLOX* gene. There was no *PgLOX* gene on chromosome 7 (Fig. 1). The molecular weights (m.wt) of the LOX proteins range from 54.1 kDa (PgLOX6a) to 105.3 kDa (PgLOX4). The lengths of the predicted products encoded by these genes vary from 480 amino acids (aa)(PgLOX6a) to 962 aa (PgLOX4) (Table 1). The prediction for PgLOX protein localization suggests it is found in the chloroplast or the cytoplasm. Five of the ten *PgLOX* genes were annotated as targeted to the chloroplast, while the remaining five were found in the cytoplasm. The isoelectric point (pI) predictions for PgLOX proteins suggest that most are acidic. However, PgLOX5a (7.2) and PgLOX6a (9.5) are predicted to be basic (Table 1). The aliphatic index of the identified PgLOX proteins ranged from 75.2 to 87.9, while the GRAVY (Grand Average of Hydropathy) values varied between −0.274 and −0.546 (Table 1). The negative GRAVY values of the PgLOXs indicate that they are hydrophilic. The exon-intron structures of the PgLOX genes were further examined. The results showed that *PgLOX5a* and *PgLOX2c* each have eight introns; three *LOX* genes (*PgLOX2b*, *PgLOX2a*, and *PgLOX3*) have six introns; three other *LOX* genes contain four introns; and one *LOX* gene (*PgLOX1*) has seven introns (Supplementary Fig. 2). Other parameters, such as gene IDs, CDS length, protein lengths, number of exons, and positions of the PLAT/LOX domains, are summarized in Table 1.

Phylogenetic Analysis of the PgLOXs

To elucidate the evolutionary relationships among PgLOX genes, a phylogenetic tree was constructed using the LOX family from rice, foxtail millet, and sorghum (Fig. 2). The ten PgLOX proteins were clustered into two main subfamilies: 9-LOX or 13-LOX. Among them, five genes (PgLOX2b, PgLOX6a, PgLOX2c, PgLOX6b, and PgLOX1) were classified as 9-LOXs, along with seven SiLOX genes (SiLOX2, SiLOX3, SiLOX6, SiLOX8, SiLOX9, SiLOX10, and SiLOX11) and six OsLOX genes (OsLOX4, OsLOX5, OsLOX7, OsLOX10, OsL2, and r9/LOX1). The 13-LOX group was further divided into two types: type I and type II. Type I lacks plastid-targeting peptides and exhibits high sequence similarity (> 75%), whereas type II contains plastid-targeting peptides and shows lower sequence similarity among its members. In pearl millet, all five remaining PgLOX genes (PgLOX5a, PgLOX5b, PgLOX4, PgLOX2a,

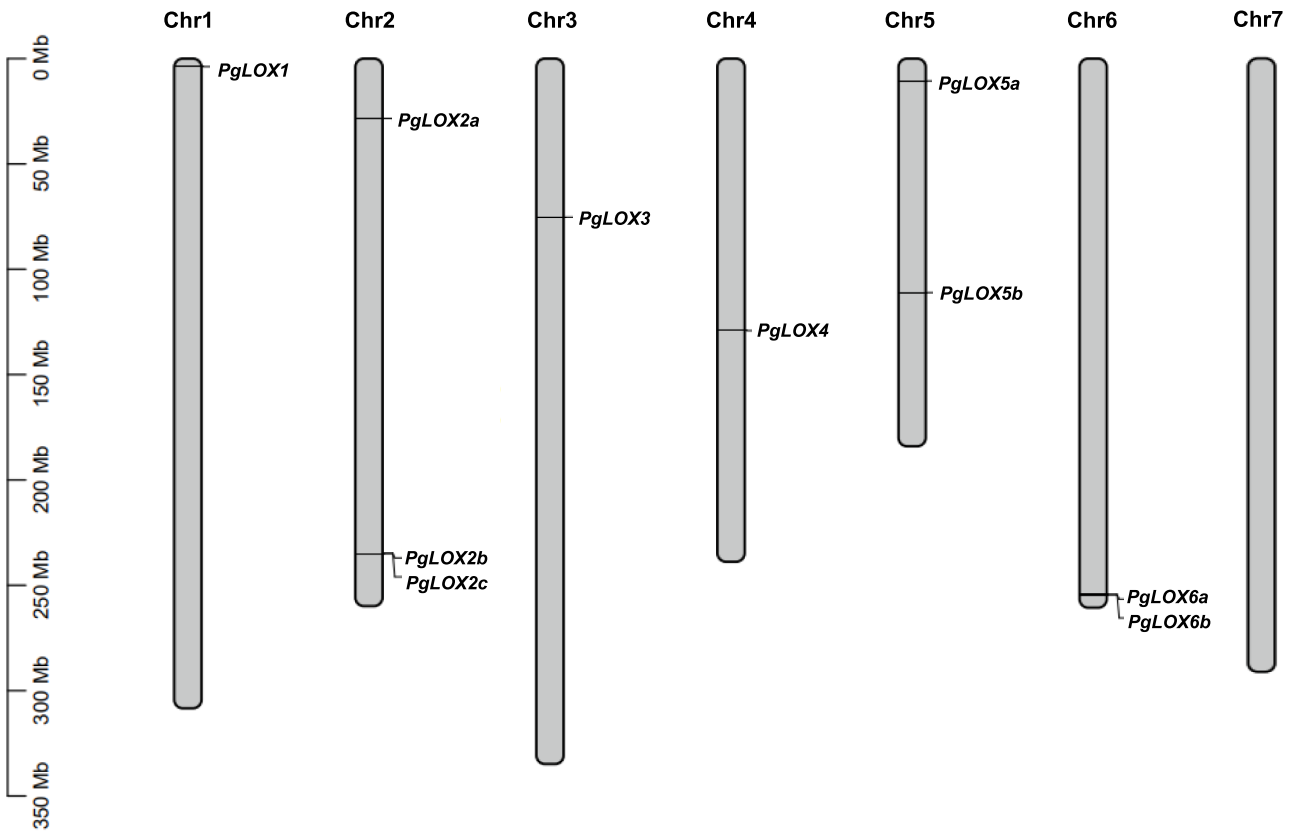

Fig. 1 Chromosome localization of *PgLOX* gene family in pearl millet. The chromosome is illustrated at the top of each bar. The length of chromosomes is shown in millions of bases (Mb) on the scale bar on the left side of the figure

and PgLOX3) were classified as 13-LOX type II. This subgroup also included five SiLOX genes (SiLOX1, SiLOX4, SiLOX5, SiLOX7, and SiLOX13) and six OsLOX genes (OsLOX1, OsLOX2, OsLOX6, OsLOX8, OsLOX11, and OsHI-LOX) (Fig. 2).

Conserved Motifs, Tertiary Structural Features, and Protein–Protein Interactions of PgLOXs

The PgLOX gene family protein sequences were analyzed for conserved motifs and domains using MEME motif analysis (Fig. 3a). Eight conserved motifs were identified among nine PgLOX proteins: PgLOX1, PgLOX2a, PgLOX2b, PgLOX2c, PgLOX4, PgLOX5a, PgLOX5b, PgLOX6a, and PgLOX6b (Fig. 3). These motifs appeared in a similar order, indicating that the positions of the motifs in PgLOX proteins are conserved. Most motifs are found in the lipoxygenase domain. Motif 1, which contains a histidine signature, an oxygen-binding domain, and a C-terminal motif, is highly conserved across all nine lipoxygenases (Fig. 3a). The amino acid sequence of PgLOX6a has three motifs: motifs 3, 4, and 6 (Fig. 3a). Functional domains are identified in PgLOX proteins; all ten PgLOX proteins contain both "lipoxygenase" and "PLAT" domains (Fig. 3a; Table 1).

The tertiary structures of the PgLOX proteins were predicted based on their homology to a template protein identified through BLAST, HHblits, or AFDB searches in the SWISS-MODEL Repository (Fig. 1). The templates used for predicting the tertiary structures of the PgLOX proteins, along with their sequence identity and similarity, are summarized in Supplementary Table 2. All PgLOX protein templates were identified using the AlphaFold v2 method, except for PgLOX1, which was predicted through X-ray crystallography. The sequence identity, which exceeded 70% (except PgLOX1), indicates high homology in the predicted models. The GMQE scores for the predicted PgLOX tertiary structures ranged from 0.74 to 0.91, suggesting high data reliability (Supplementary Fig. 3; Supplementary Table 2).

The STRING database also created a protein–protein interaction network for PgLOX proteins. This network comprised 57 edges representing interactions, with an average node degree of 8.77 and an average local clustering coefficient of 0.715. The protein–protein interaction enrichment *P*-value was less than $< 1.0e\text{-}16$, $< 1.0e\text{-}16$, indicating a significant interaction among the 13 involved nodes. Eight

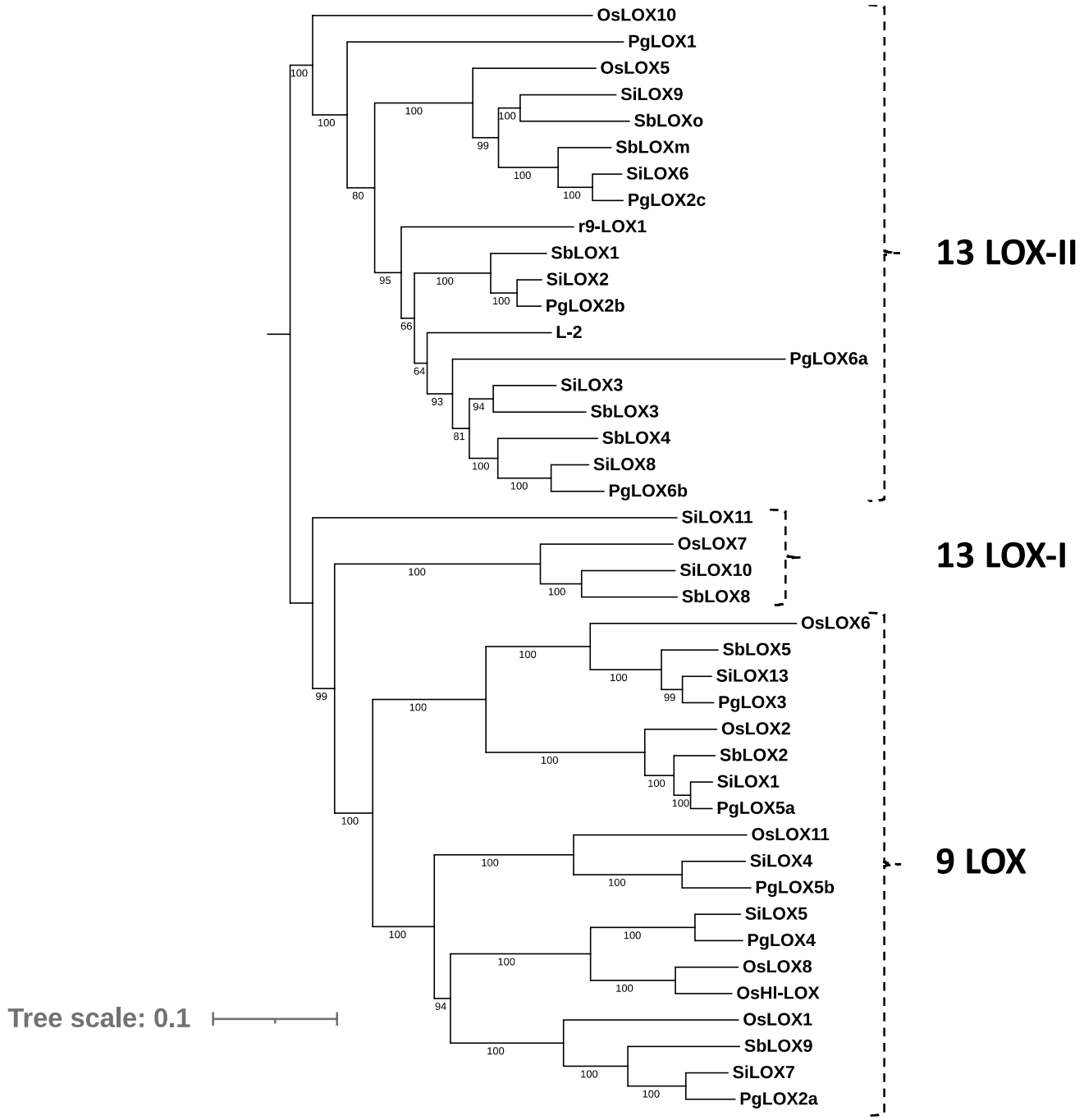

Fig. 2 Phylogenetic tree of LOX proteins from *Pennisetum glaucum*, *Oryza sativa*, *Setaria italica*, and *Sorghum bicolor*. The multiple sequence alignment was performed using the default parameters for ClustalW in Mega 11.0, where different clades are labelled according to the category

PgLOXs were identified as interacting, with PgLOX2a and PgLOX4 showing the highest interactions (11 each) with PgLOXs and other proteins. In contrast, PgLOX5b showed the lowest interaction, linking with only six proteins. Further STRING analysis expanded the network to include non-LOX proteins, resulting in 13 proteins, including 8 PgLOXs. Interactions were observed with proteins such as PLA2-III (Phospholipase A2-homolog), Q2RAM0_ORYSJ (a lipase family protein), and SLM1 (a cytochrome P450 family protein). Notably, all PgLOXs are predicted to interact with PLA2-III, which plays a role in the calcium-dependent hydrolysis of phosphoglycerides, releasing lysophospholipids (LPLs) and free fatty acids (FFAs) in response to various stimuli. The protein interaction network revealed potential interactions between PgLOX proteins and enzymes such as allene oxide synthase 1 (CYP74A1) and allene oxide synthase 3

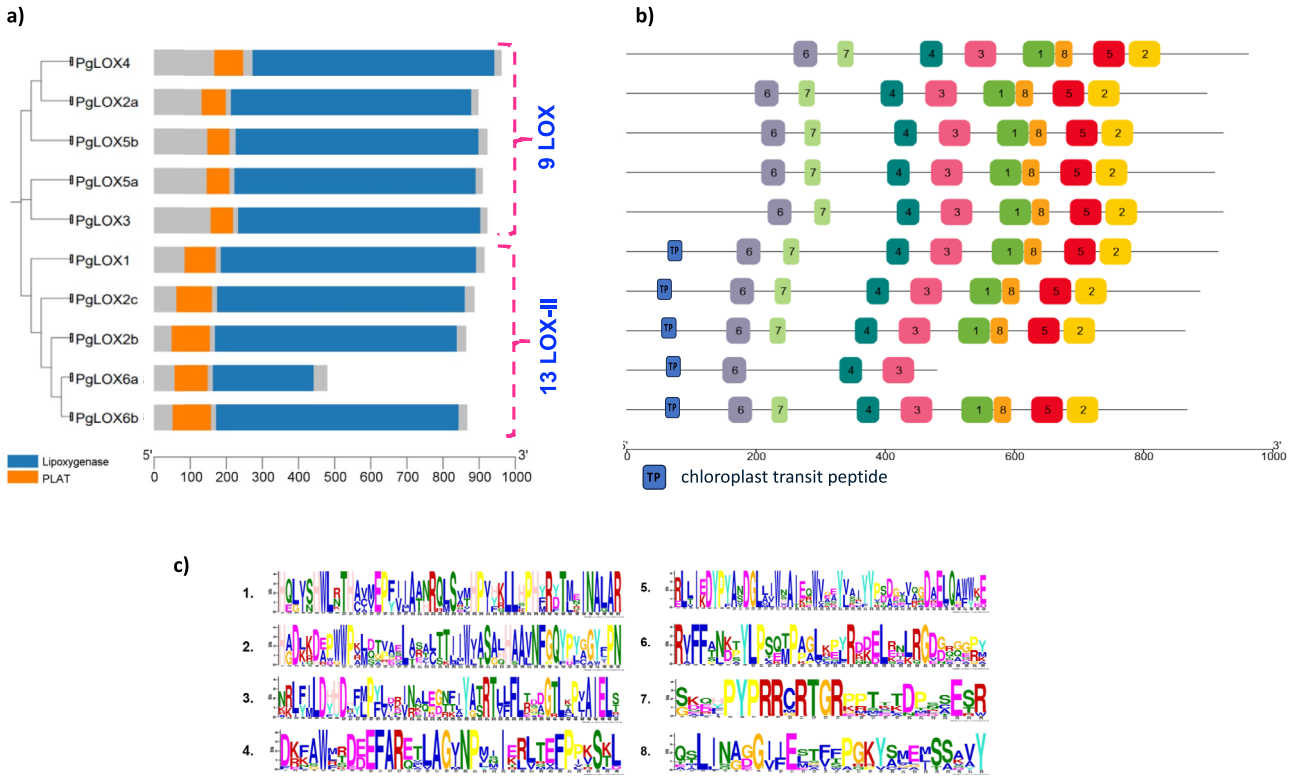

Fig. 3 Conserved domains and motifs prediction of PgLOX proteins. **a** Protein-conserved domains were identified using the NCBI-CDD tool. PLAT and Lipoxygenase domains are shown in orange and blue, respectively. **b** Conserved motif positions identified in PgLOX proteins using the MEME online tool are displayed in different colors. **c** Conserved motif logos identified in PgLOX proteins

(CYP74A3), both of which are known for their role in jasmonic acid biosynthesis (Supplementary Fig. 5; Supplementary Table 3).

Synteny, Ka/Ks, and Divergence Time Analysis of *PgLOX* Genes

Synteny analysis was conducted to elucidate the evolutionary relationships of LOXs in pearl millet compared to other cereal crops. A collinearity syntenic map included pearl millet, rice, sorghum, and foxtail millet (Supplementary Fig. 4). Notably, PgLOX2b and PgLOX6a show collinearity across all three crops: rice (LOC_Os03g49350 and LOC_Os02g10120), sorghum (Sobic.001G125700 and Sobic.004G078600), and foxtail millet (Seita.9G127600 and Seita.4G215400). A distinct collinear pair of pearl millet PgLOX6b was identified between sorghum (Sobic.003G385500) and foxtail millet (Seita.5G411600).

To assess if selection pressure influenced the evolution of the PgLOXs, we calculated the ratio of non-synonymous (Ka) to synonymous (Ks) replacement rate using TBtools. Three duplications were observed in the PgLOXs: one tandem duplication (PgLOX6a/PgLOX6b) and two segmental duplications (PgLOX2a/PgLOX5b and PgLOX3/PgLOX5a). This indicates that the PgLOX family members have expanded through segmental duplication events, with predicted divergence time ranges between 34.223719 and 99.3757851 million years ago (Mya) (Supplementary Table 4). The Ka/Ks ratios of paralogs were below 1, indicating purifying selection in the PgLOXs, similar to other millets. This highlights the role of environmental pressure during evolution. The results suggest that purifying selection stabilizes the conserved structural and functional roles of the PgLOX family members. Eight orthologous events between pearl millet and foxtail millet suggest an evolutionary relationship. The Ka/Ks values range from 0.2102 to 1.0878. Most gene pairs exhibited purifying selection, but two pairs, PgLOX2a/SiLOX7 with a value of 1.0643 and PgLOX6b/SiLOX8 with a value of 1.0878, show positive selection pressure (Supplementary Table 5). The estimated divergence time between PgLOX and SiLOX orthologs ranges from 6.08215189 to 87.9952103 million years ago (mya) (Supplementary Table 5).

Cis-Acting Elements Prediction in the Promoter of *PgLOX* Genes

The *cis*-acting elements of the *LOX* gene promoter regions were analyzed to understand their functions and regulation.

A comprehensive analysis identified these *cis*-acting elements within the 1000 bp promoter region of *PgLOX* genes. The predicted elements were categorized into four main groups: light-responsive, hormone-responsive, stress-responsive, and plant growth and development. The G-box element was the most common light-responsive element found in all *PgLOX* gene promoter regions, with *PgLOX6b* containing the highest number of G-box elements (Fig. 4). Regarding phytohormone-responsive elements, the *PgLOX* gene promoters included those that respond to Methyl jasmonate (MeJA), Abscisic acid (ABA), Salicylic acid (SA), Gibberellic acid (GA), and Auxin. Abscisic acid-responsive elements (ABRE) were abundant in all *PgLOX* promoters except for *PgLOX6a*. Additionally, MeJA-responsive

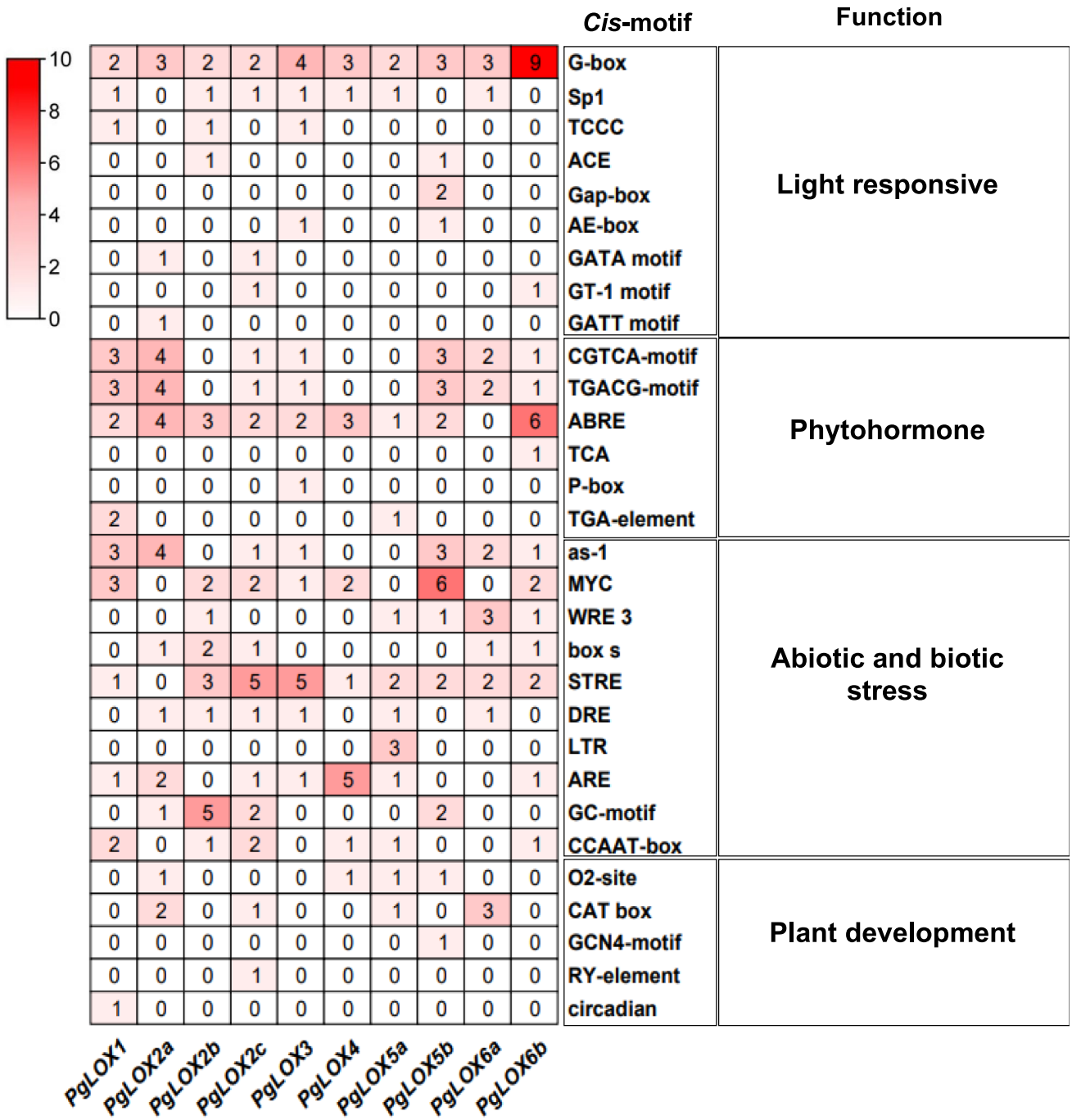

Fig. 4 The distribution of cis-acting elements in the putative promoter of *PgLOX* genes. The figure represents various cis-acting elements labelled with different colors. The scale is represented on the left

elements (CGTCA-motif and TGACG-motif) were abundant, ranking as the second most prevalent. Interestingly, SA-responsive elements (TCA-element), Auxin-responsive elements (TGA-element), and GA-responsive elements (P-Box) were observed exclusively in the promoter regions of *PgLOX6b*, *PgLOX1*, and *PgLOX3*, respectively (Fig. 4). For stress-responsive elements, the most prominent ones responding to drought include as-1, Myc, and the stress-responsive element STRE. Additionally, elements responsive to anaerobic induction (ARE) and anoxic-specific inducibility (GC motif) were evident in the *PgLOX* promoters. Notably, a low-temperature responsive element (LTR) was exclusively identified in the promoter region of *PgLOX5a* (Fig. 4). Regarding plant growth and regulation-related elements, unique elements specific to the promoter regions of certain genes were observed. These include the GCN4 motif, which regulates endosperm-specific expression in the promoter region of *PgLOX5b*; the RY-element, responsible for seed-specific regulation in the promoter region of *PgLOX2c;* and an element that regulates the circadian clock in the promoter region of *PgLOX1* (Fig. 4). These results suggest that the *PgLOX* genes may play a role in growth, development, and response to light, hormones, and stress. The exact position of the cis-element is shown in the Supplementary Table 6.

Expression Analysis of the *PgLOX* Genes

The expression levels of the *PgLOX* genes were evaluated in the grain and leaf tissue using qRT-PCR across two pearl millet genotypes that differ in rancidity. In grain, *PgLOX1* showed significantly higher expression in the high rancid genotype (ICMB863) compared to the low rancid genotype (ICMB95222). In contrast, *PgLOX2b* showed significantly higher expression in the grain of the low rancid genotype (ICMB95222) than the high rancid genotype (ICMB863) (Fig. 5). The expression of *PgLOX6a* in the grain was insignificant in either the high or low rancid genotypes (Fig. 5). The remaining candidate genes, including *PgLOX2a*, *PgLOX2c*, *PgLOX3*, *PgLOX4*, *PgLOX5a*, *PgLOX5b*, and *PgLOX6b*, were not expressed in the grain. Additionally, we analyzed the expression levels of selected *PgLOX* genes (*PgLOX1*, *PgLOX2b,* and *PgLOX6a*) in leaf tissue. Interestingly, the *PgLOX1* gene was not expressed in the vegetative tissue (Leaf) of both high- and low-rancid genotypes (Supplementary Fig. 7). In contrast, *PgLOX2b* and *PgLOX6a* were expressed in the grain (Fig. 5; Supplementary Fig. 7).

PgLOX1 Gene Co-expression Network and Functional Enrichment Analysis During Grain Development

Co-expression analysis of *PgLOX1* using RNA-seq data from pearl millet panicles exposed to high temperatures identified 8 negatively and 60 positively correlated genes ($r \geq 0.6$) (Lou et al. 2024). The co-expression network visualized in Cytoscape (Supplementary Fig. 6A) highlighted strong links with lipid metabolism, stress signaling, and secondary metabolic processes. Among the top positively correlated genes were calcium-dependent protein kinase 3-like, alpha-galactosidase-like, putative linoleate 9S-lipoxygenase 3, LOX4, and retinol dehydrogenase 12-like (Supplementary Table 7). Negatively

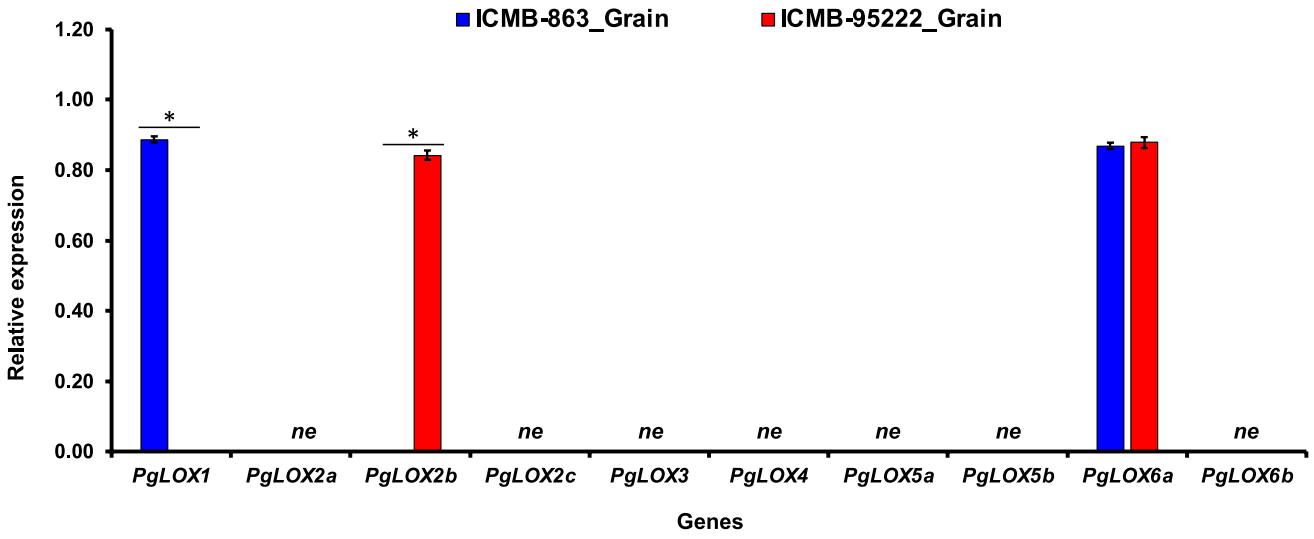

Fig. 5 Expression analysis of ten *PgLOX* genes in the grain across two contrasting pearl millet genotypes using qRT-PCR. Expression levels were evaluated in the grain (fresh flour) in both genotypes. Levels were normalized using the reference gene *PgEIF4a*. Significant differences ($P < 0.05$) were noted in between-group comparisons, as indicated by asterisks in the bar diagrams. The symbol "ne" indicates that the gene is not expressed

correlated genes included E3 ubiquitin-protein ligase MARCH5-like, glucan endo-1,3-beta-glucosidase 8-like, UDP-N-acetylglucosamine pyrophosphorylase, ZINC INDUCED FACILITATOR-LIKE 1 isoform X1, and 65 kDa microtubule-associated protein 6-like (Supplementary Table 7). Functional enrichment analysis with MilletDB revealed major KEGG pathways such as linoleic acid metabolism, alanine, aspartate, and glutamate metabolism, galactose metabolism, amino sugar and nucleotide sugar metabolism, carotenoid biosynthesis, and alpha-linolenic acid metabolism. GO terms were enriched for oxidoreductase activity, linoleate 13S-lipoxygenase activity, actin cytoskeleton organization, ubiquitin ligase complex, and protein ubiquitination (Supplementary Fig. 6B-C). A complete list of correlated genes and enrichment results is available in Supplementary Table 7.

Discussion

Climate change poses a major threat to sustainable agriculture, affecting vital crops like wheat, maize, and rice. Declines in yields and productivity (Sharma et al. 2022) of these staples directly cause food shortages and hunger, as they are essential for global food security. Cultivating nutritious crops that thrive in extreme climatic conditions is necessary. Pearl millet provides an excellent solution to these challenges (Satyavathi et al. 2021). As a climate-resilient crop, it is well suited for drought-prone areas and has a strong nutritional profile, offering a rich energy source and essential nutrients. It is high in polyunsaturated fatty acids (PUFA) (Singh et al. 2018), indicating oleic and linoleic acids, which are important for enzyme activity.

Pearl millet excels in many ways; however, its global consumer acceptance requires enhancement due to its short shelf life of milled flour. Enzymes play a crucial role in causing rancidity in milled flour. These include lipases, lipoxygenases (LOX), peroxidases, and polyphenol oxidases (PPOs). *LOX* genes are part of the Fe or Mn family and contain dioxygenase, which oxidizes polyunsaturated fatty acids. Immediately after grinding, highly active lipases break down triacylglycerols into monoacylglycerols, diacylglycerols, and free fatty acids (FFA) (Aher et al. 2022). These FFAs serve as substrates for LOX activity, which produces primary oxidative products such as hydroperoxides (Vinutha et al. 2022). Subsequently, POX enzymes generate secondary oxidative products, including aldehydes and ketones. These secondary products cause the off-flavor and smell of pearl millet flour (Goswami et al. 2020).

Recently, researchers have been studying the biochemical and molecular mechanisms involved in flour rancidification (Aher et al. 2022). Aher et al. (2022) identified differences in two TAG lipases in pearl millet, which may help protect lipids from hydrolysis. Rancidity is a complex trait that needs a comprehensive and integrated approach to understanding the mechanisms and pathways for identifying specific candidate genes. In this study, we characterized the *LOX* gene family in pearl millet, helping researchers identify candidate *LOX* genes involved in fatty acid oxidation. Using the high-quality genome database Milletdb (Sun et al. 2023), we identified ten *PgLOX* genes, further verified through conserved domains (Table 1). The number of identified LOX genes in pearl millet is fewer than in other monocot plant species, such as rice (14) (Umate 2011) and maize (13) (Ogunola et al. 2017), but greater than in sorghum (9) (Shrestha et al. 2021). Although LOX proteins share similar biochemical functions across plant species, the different numbers of these genes indicate they are not conserved during evolution (Shrestha et al. 2021).

Gene duplication and loss are essential for expanding gene families and creating functional variation (Sampedro et al. 2005). To better understand the evolutionary relationships among the identified PgLOX proteins, we constructed a phylogenetic tree that includes other monocot species, such as rice, foxtail millet, and sorghum. In pearl millet, one tandem duplication and two segmental duplications were observed, indicating that the *PgLOX* gene family may expand through segmental duplications. Tandem and segmental duplications contribute to expanding gene families (Cannon et al. 2004). The gene duplication events and their Ka/Ks ratios indicate that the PgLOXs evolved under purifying selection. This explains why there are fewer LOXs in this crop compared to others. Purifying selection preserves their native and ancestral functions by eliminating harmful mutations. Eight orthologous events were observed between pearl millet and foxtail millet, indicating a close evolutionary relationship. Synteny analysis supports this finding by demonstrating their shared evolutionary development. Eight orthologous events between pearl millet and foxtail millet indicate a close evolutionary relationship, supported by synteny analysis. All the identified paralogs occurred in these orthologous events, suggesting their functional maintenance.

Furthermore, the study of gene duplications, rearrangements, and evolution shows that pearl millet and foxtail millet shared a common ancestor. The whole genome duplication event in the Poaceae family, which includes *Pennisetum*, occurred approximately 70 million years ago (Wang et al. 2011). The analysis of divergence times for duplicated genes aligns with the timing of this event.

Phylogenetic analysis indicated that PgLOX proteins can be divided into 9-LOX and 13-LOX (Fig. 2). This classification aligns with studies on sorghum (Shrestha et al. 2021), foxtail millet (Zhang et al. 2021), soybean (Zhang et al. 2022), banana (Liu et al. 2021), and poplar (Chen et al. 2015). Accordingly, we identified five members in the 13-LOX group (PgLOX2a, PgLOX3, PgLOX4, PgLOX5a,

PgLOX5b) that belong to the type II group, exhibiting moderate to low sequence similarity and containing a chloroplast transit peptide. In contrast, proteins in the 9-LOX group (PgLOX1, PgLOX2b, PgLOX2c, PgLOX6a, PgLOX6b) display high sequence similarity and lack a chloroplast transit peptide. According to the classification system proposed by Feussner and Wasternack (2002), Type I *LOX* genes share a significant sequence similarity of over 75% and lack a chloroplast transit peptide. In contrast, type II LOXs have moderate overall sequence similarity of up to 35% and include a chloroplast transit peptide (Feussner and Wasternack 2002).

Additionally, motif analysis of the amino acid sequences of the identified PgLOX proteins shows that all eight predicted motifs are conserved in most PgLOX proteins. This highlights both sequence and functional conservation (Fig. 3). These conserved motifs indicate their potential importance in maintaining the structure and function of PgLOXs. The molecular function of biological molecules is closely related to their molecular structure (Nguyen et al. 2011). The tertiary structures of PgLOX proteins identified in this study provide a basis for understanding their exact biological functions (Supplementary Fig. 3). An analysis of the *cis*-acting elements in the promoter region of *PgLOX* genes revealed many light-responsive elements (Fig. 4). These results indicate that light influences the activity of LOX enzymes. Previous literature suggests that excessive red light can induce *LOX* gene activation during a plant's defence response, a process mediated by phytochrome B (Zhao et al. 2014; Ji et al. 2020). LOX enzymes play a crucial role in the biosynthesis of jasmonic acid (Wang et al. 2021), supported by a protein–protein interaction network that predicts an interaction between PgLOX proteins and both allene oxide synthase 1 (CYP74A1) and allene oxide synthase 2 (CYP74A2), which are involved in the jasmonic acid biosynthesis. In *Arabidopsis*, the expression of LOX is well-documented as being triggered by jasmonic acid (JA) due to a positive feedback loop that amplifies JA responses (Hickman et al. 2017). Identifying the elements responsible for MeJA in the promoter regions of *PgLOX* genes suggests that this feedback loop is conserved across species (Fig. 4). JA also regulates responses to plants' biotic and abiotic stresses (Ghorbel et al. 2011; Raza et al. 2021). As one of the enzymes in the biosynthetic pathway of JA, we identified various biotic and abiotic stress-responsive elements in the promoter regions of *PgLOX* genes. The interaction of PgLOX proteins with Q2RAM0_ORYSJ, an alpha-dioxygenase protein involved in fatty acid oxidation, identified through protein–protein interactions (PPI), indicates a role in lipid oxidation and hydrolysis, ultimately leading to rancidity (Aher et al. 2022; Satyavathi et al. 2021). *PgLOX1* is connected to lipid metabolism, calcium signaling, and ubiquitin regulation, serving as a crucial lipid signaling center in pearl millet panicles under heat stress. By boosting lipid oxidation and oxylipin production, *PgLOX1* aids heat adaptation and helps keep seed stability. Its positive co-expression with calcium-dependent kinases and other lipoxygenases indicates coordinated stress responses, while its negative associations with ubiquitin ligases and cell wall enzymes suggest metabolic regulation. Overall, these diverse roles make *PgLOX1* a promising target for breeding heat-tolerant, high-quality millet cultivars (Supplementary Fig. 7).

Aher et al. (2022) identified line ICMB863 as highly rancid due to a higher accumulation of free fatty acids (FFA), the primary products of lipoxygenase (LOX) activity. *LOX* genes regulate ripening and senescence, helping synthesize ethylene and oxidize PUFA to produce superoxide radicals that disrupt cell membrane structure (Han et al. 2011; Zhang et al. 2014). The literature suggests that specific LOX proteins, such as LOX2 and LOX3, have a greater affinity for TAG and methyl esters of fatty acids than FFA (Erba et al. 2024). *LOX* genes are known to be expressed in seed tissue. For example, *r9-LOX1* and *L-2* showed increased expression levels in active rice bran and seeds compared to stabilized and mature seeds (RoyChowdhury et al. 2016). In soybeans, *GmLOX1, GmLOX2*, and *GmLOX3* exhibited higher expression in seeds. *PgLOX1* also showed increased expression in the grain of the high rancid line ICMB863 and was absent in leaf tissue. This may play a crucial role in fatty acid oxidation, following a similar mechanism that leads to rancidity, which can affect grain quality (Fig. 5; Supplementary Fig. 7).

Furthermore, when comparing the expression of these genes with grain, *PgLOX1* reveals a unique expression pattern specific to the high-rancid genotype (Fig. 5). Given the identification and expression analysis of *LOX* genes in pearl millet, it is essential to further investigate the role of *PgLOX1* in regulating rancidity and grain quality through overexpression and knockout experiments. This study's findings provide a basis for further exploration of the role of *LOX* genes in rancidity.

Conclusions

Ten *PgLOX* genes were identified from the pearl millet genome, and their sequence characteristics—including phylogenetic relationships, conserved functional motifs, protein networks, gene duplication events, and *cis*-regulatory elements—were comprehensively analyzed. Members of the LOX subfamilies exhibited conserved gene structures and motif compositions. Promoter analysis indicated the presence of complex, developmentally regulated cis-elements that may govern *PgLOX* gene expression. Expression profiling revealed that *PgLOX1* was highly expressed in grains, suggesting its potential role in determining the grain quality

and in regulating downstream processes such as rancidity development.

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Author Contribution Conceptualization, W.T. and P.S.R.; methodology, P.S.R. and P.R.B.; software, P.S.R.; validation, P.S.R. and P.R.B.; formal analysis, W.T. and P.S.R.; investigation, P.S.R. and P.R.B.; data curation, W.T. and P.S.R.; writing—original draft preparation, P.S.R.; writing—review and editing, W.T. and P.S.R.; visualization, P.S.R. and P.R.B.; supervision, W.T.; project administration, W.T.; funding acquisition, W.T.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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