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Genome-Wide Identification and Expression Analysis of *LOX* Gene Family in Rice (*Oryza sativa* L.) Under Abiotic Stress Conditions

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Abstract

Background: Lipoxygenases (*LOXs*) are enzymes that facilitate the
oxygenation of polyunsaturated fatty acids (PUFAs) to create oxylipins
through hydroperoxides that are involved in methyl jasmonate (MeJA)

22 signaling pathway and are essential for seed germination, growth and
23 development, defense mechanisms, and responses to stress. This study
24 was designed for systematic analysis and exploration of expression
25 patterns of the *LOX* gene family of rice. **Results:** Twelve *LOX* genes were
26 identified, and phylogenetic and structural analyses grouped them into 9-
27 LOXs, Type I 13-LOXs, and Type II 13-LOXs subfamilies with conserved
28 lipoxygenase domains, motif and gene structure patterns. These genes
29 were dispersed unevenly across seven chromosomes. Analysis of gene
30 duplication found four tandem and segmental duplication events
31 predominantly driving their moderate expansion, with Ka/Ks ratios
32 suggesting purifying selection. Syntenic analysis across three genomes
33 indicated relatively conserved roles of orthologous gene pairs of this
34 family. The cis-acting regulatory elements revealed 46 CRE motifs
35 responsive to light, cellular development, hormones and stress. Notably,
36 151 putative miRNAs were identified as potential post-transcriptional
37 regulators. PPI analysis highlighted 10 key nodes with high interaction
38 degrees. GO enrichment classified the functions of *OsLOXs* into biological,
39 metabolic and cellular processes. RNA-seq expression profiling
40 demonstrated *OsLOXs* expression across diverse tissues, developmental
41 stages and stress. RT-qPCR data analysis further validated that *OsLOX*
42 genes exhibited temporally variable differential expression under multiple
43 abiotic stress conditions including drought, salinity, saline-alkalinity, heat
44 and cold, and in response to IAA hormonal treatment. **Conclusion:** In
45 conclusions, drought and cold stress significantly upregulated certain
46 *OsLOXs*, enabling rice plants to confront these adverse conditions. This

study will underscore the path for forthcoming in-vivo analyses to uncover the molecular processes of *LOX* genes involved in tolerance to stress in rice.

Keywords: *LOX*, Phylogeny, Cis-elements, Expression profiling, GO enrichment, PPI, Abiotic stress, RT-qPCR

Background

Rice (*Oryza sativa*) is considered as the preeminent cereal grain globally, feeding above 50% of the earth's population [1]. Multitude of abiotic challenges resulting from environmental and climate changes such as salinity, drought, submergence, extreme temperatures, nutrient deficiencies and heavy metal accumulation [2], severely disrupt physiological [3], biochemical [4], molecular [5], and metabolic processes in rice plants [6], thereby adversely impacting their growth and development, productivity, and overall, an annual yield reduction of 32% (approximately 3 million tons) worldwide [1]. Abiotic stress response in rice plants occurs via induction of complex gene network modules [7], and the adaptation in changing environmental situations is well-regulated by the perception and stress signals transduction [8]. Rice is the first species to have its entire genome sequenced among cereal grains. Initial draft genomes for the *japonica* and *indica* subspecies (regarded as 93-11) were released in 2002. Although, the *Japonica* cultivar *Nipponbare* was the inaugural high-quality reference genome for rice, and indeed for any cereal species, which was released in 2005 [9]. Several studies have

identified functionally annotated and validated expression profiles of stress responsive genes in rice through the analysis of genomes and transcriptomics data [10–12]. Therefore, characterizing the genetic background of rice thorough a genome wide investigation of stress-responsive families is essential for elucidating molecular mechanisms of stress tolerance and for breeding varieties with high adaptability under environmentally constrained conditions [11,13,14].

Lipoxygenases (LOXs) are an enzyme that is present in both plants and mammals. They are members of the iron-containing fatty acid dioxygenase family and include linoleate: oxygen oxidoreductase (EC 1.13.11.12) [15]. It enables the conversion of polyunsaturated fatty acids (PUFAs) from plants into linoleic acid (LA, C18:2 ω 6), linolenic acid (ALA, C18:3 ω 3), and arachidonic acid (AA, C20:4 ω 6) through the process of oxygenation and dehydrogenation. This process results in the formation of unsaturated hydroperoxides [16]. These intermediate products undergo several metabolic reactions to generate oxygenated derivatives including jasmonic acid (JA), reactive oxylipins containing epoxides, aromatic compounds conjugated carbonyls or aldehydes, and leaf aldehydes and diethylene ethers which have antibacterial and antifungal activities [17]. Additionally, certain PUFAs are metabolized by α -dioxygenase (α -DOX) enzymes to produce α -hydroxy or α -peroxy PUFAs [18].

Two spatially separated LOX pathways: 9-LOX and 13-LOX have been observed in flora. 13-LOX pathway was classified further as Type I 13-LOX and Type II 13-LOX, present on cytoplasm and chloroplast respectively

95 [16,19]. The N-terminal of *LOX* gene contains a conserved PLAT/LH2
96 (polycystin-1, lipoxygenase, alpha-toxin/lipoxygenase homology) domain,
97 while the C-terminal of true *LOX* genes contains a characteristics
98 lipoxygenase domain composed of histidine amino acid rich region [His-
99 (X)4-His-(X)4-His-(X)17-His-(X)8-His] [20]. Numerous signal molecules are
100 generated via LOX-mediated pathways; among these, the major signalling
101 compounds are C6-volatile compounds and jasmonates which contribute
102 to plant physiological responses encompass germination [21], growth and
103 development [22], fruit ripening [23], reactions to both biotic and abiotic
104 stressors [24-28], wounding [29], senescence and cell death [30], and
105 synthesis of stress responsive hormones such as jasmonic acid (JA) and
106 abscisic acid (ABA) [31,32]. Plants express numerous LOX genes during
107 stress, which induces physiological and anatomical modifications to
108 accommodate adverse conditions [33]. *LOX* is a polygenic family studied
109 and characterized in several plants. Understanding the specific
110 physiological function of *LOX* genes has been challenging given the
111 prevalence of several isozymes [34]. The occurrence of these distinct *LOX*
112 isoforms is contingent upon the tissue, sub-cellular spaces, and stages of
113 embryogenesis, and their catalytic features, tissue-specific gene
114 expression, and amino acid sequences vary [35]. Prior research has
115 determined a cumulative sum of 6 *LOX* family members in *Arabidopsis*
116 [36], 36 members in soybean [37], 11 members in radish and tea tree
117 [38,39], 12 members in *Foxtail millet* [40], 18 members in melon [41], 14
118 members in strawberries [42], 20 members in *poplar* and *Artemisia annua*
119 and so on. Among many C3 photosynthetic cereal crops, durum wheat has

been shown to upregulate *TdLpx-A2* during hyperosmotic stress and thereby counteract excess ROS generation by *TdLOX2* to mitigate plant oxidative damage [43]. CRISPR/Cas9-mediated overexpression line of soybean demonstrated *GmLOX6* increases salinity tolerance through JA-mediated biosynthesis pathway [44]. C4 model plant foxtail millet exhibited a substantial upregulation of *SiLOX7* during the exposure of saline condition in two stress-tolerant varieties [40]. In oriental melon, suppression of *CmLOX10* has been demonstrated to enhance drought susceptibility via jasmonic acid-mediated stomatal closure and feedback involving *CmMYC2* [27]. Cloning of the computationally identified promoter region of *CmLOX08* elucidated the mechanisms underlying resistance to abiotic stresses through signaling molecules and stress-inducible core promoters [45]. Further study on *Medicago truncatula* confirmed that exogenous *MtLOX24* overexpression in *Arabidopsis* mitigates the MeJA-induced oxidative damage, suggesting its role to insect related abiotic stress responses (L. Xu et al., 2024).

Although numerous research has examined rice transcriptome data and co-expression patterns in response to various abiotic and biotic challenges, a systematic examination of the *LOX* gene family and its specific response to abiotic stress remains lacking. A comprehensive characterization and expression profiling of *LOX* gene family of rice may offer significant understanding of its function under abiotic stressors, attributable to the presence of lipoxygenase enzymes. Thus, our approach can effectively identify and characterize *LOX* gene family, by uncovering their evolutionary relationships, expression profiles, and potential

functionalities. Additional, functional characterization of selective *LOX*s in response to abiotic and hormonal stress will yield insights for further examination of rice *LOX* gene family.

Materials and methods

Sequence curation of Lipoxygenase genes in *Oryza sativa* genome and physicochemical properties analysis

A multi-step method employing diverse bioinformatics resources and databases was applied to discover *LOX* family members in rice. TAIR database (<https://www.arabidopsis.org/>) [47] was utilized to obtain the protein sequences for six *Arabidopsis thaliana* genes (*At1g55020*, *At3g45140*, *At1g17420*, *At1g72520*, *At3g22400*, *At1g67560*) that are members of *lipoxygenase* gene family [48]. The rice LOX proteins were identified by undertaking a BLASTP search against the *Oryza sativa* v7.0 genome dataset in the Phytozome database (https://phytozome-next.jgi.doe.gov/info/Osativa_v7_0) [49]. For the sake of comprehensive identification, these AtLOX protein sequences were additionally tested against the Rice Genome Annotation Project (RGAP) database (<https://rice.uga.edu/>) [50] and NCBI protein database (<https://www.ncbi.nlm.nih.gov/protein>) [51] individually to ensure no potential member was overlooked. To identify all the possible *LOX* genes, Hidden Markov Model (HMM) profile for Lipoxygenase domain (PF00305) and PLAT/LH2 domain (PF01477) were acquired from the Pfam database [52] with HMMER program (v3.4) [53]. The e-value threshold was established at $<1e-5$. To further validate the presence and integrity of

conserved domains, the presumed *LOX* sequences were analyzed using SMART (<http://smart.embl-heidelberg.de/>) [54], NCBI CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) [55], and InterPro (<http://www.ebi.ac.uk/interpro/>) [56]. The genes were renamed as '*OsLOX*' with the '*Os*' prefix indicating *Oryza sativa* and sequentially numbered according to chromosomal positions (from top to bottom), following an established naming procedure [57]. Gene lengths, CDS lengths, locus IDs, strand locations, and coding sequence (CDS) coordinates (5' to 3') were all taken from the Phytozome database [49]. Further, to ascertain similarities in the protein sequences of *OsLOX*s, multiple sequence alignments were conducted using the ClustalW alignment function of MEGA 11 [58] and results were visualized using the software GeneDoc v.2.7 [59].

The physicochemical properties of the genes, including the theoretical isoelectric point (pI), index of instability, grand average of hydropathy (GRAVY), and molecular weight of the proteins were assessed using *OsLOX* protein sequences in ProtParam hosted by ExPASy (<https://web.expasy.org/protparam/>) [60]. In addition, for determining the proteins subcellular distribution, the protein sequences FASTA file were uploaded to WoLF PSORT (<https://wolfpsort.hgc.jp/>) [61] and CELLO v.2.5 (<http://cello.life.nctu.edu.tw/>) [62]. TBtools version v2.225 [63] was employed to visualize the anticipated protein signals of each gene.

Phylogenetic relationship analysis

To generate the tree of evolutionary relationship of LOX proteins from several plant species, the completely discovered sequences of LOX proteins of *Arabidopsis thaliana* [36], *Zea mays* [64], *Brassica rapa* [65], *Sorgum bicolor* [66], *Setaria italica* [40] and *Glycine max* [37] were used to keep the rate uniform across sites. Peptide sequences of *LOX* genes of *Zea mays* (B73 reference genome), *Sorgum bicolor*, *Setaria italica* and *Glycine max* were acquired from Phytozome [49] and Brassicaceae database (<http://brassicadb.cn>) [67]. TAIR database [47] was employed for retrieval of peptide sequences of *LOX* genes from *Brassica rapa* and *Arabidopsis thaliana* genome correspondingly. By employing the MUSCLE alignment and the Maximum Likelihood Method with a 1000 bootstrap value to substantiate branch values, an unrooted phylogenetic tree was constructed in MEGA 11 [58]. The tool iTOL (<https://itol.embl.de/>) [68] was employed for tree annotation, manipulation and visualization.

Gene structure and conserved motif analysis

The organization of exon-intron of *OsLOXs* were efficiently identified and graphically depicted utilizing Gene Structure Display Server (GSDS2.0) (<http://gsds/gao-lab.org>) [69] by comparing the coding sequences (CDS) and genomic sequences lacking the UTR (untranslated region) as input resources on the server. MEME Version 5.5.8 (Multiple Em for Motif Elicitation; <https://meme-suite.org/meme/tools/meme>) [70] was utilized to identify conserved motifs on proteins, employing default parameters with the exception of selecting a maximum of 15 motifs. MEME online interface used the motif scanning method (MSA) to visualize the motifs. The role of

216 each of the 15 detected motifs was determined by evaluating them using
217 Pfam [52].

218 **Chromosomal distribution analysis**

219 MapChart software[71] was employed to create a rudimentary
220 chromosomal distribution diagram of *OsLOX* genes based on their location
221 across the 12 rice chromosomes. The details on gene loci, length for the
222 physical map and CDS coordinates obtained from the Phytozome [49] were
223 employed for pinpointing the location and position on the map.

224 **Prediction of gene duplication and collinearity analysis**

225 For estimating the divergence time of *OsLOX* genes and investigating
226 evolutionary patterns, the non-synonymous (Ka) and synonymous (Ks)
227 substitution rates for duplicated *OsLOX* gene pairs were calculated using
228 TBtools version v2.225 [63]. Segmental duplications were defined as
229 instances in which gene pairs exhibited more than 90% sequence
230 similarity, while tandem duplications were defined as two or more
231 homologous genes within a 100 kb region on the same chromosome [72].
232 Selection was inferred from Ka/Ks ratios, and divergence time was
233 determined using the formula $T = Ks/2\lambda \times 10^{-6}$ (million years). λ is defined
234 as a constant rate of 1.5×10^{-8} substitutions per site per annum for plants
235 with dicotyledons [73]. Multiple Collinearity Scan Toolkit (MCScanX) [74]
236 was used to detect gene duplication events. The *Oryza sativa* genomic
237 sequence and GFF annotation file were utilized for self-alignment in the
238 investigation of intraspecific collinearity. Interspecific collinearity analysis
239 involved comparing the genomic sequences and GFF annotation files of

240 *Arabidopsis thaliana* and *Zea mays* with the genome sequences of *Oryza*
241 *sativa*. The syntenic relationship was then determined using the syntenic
242 analysis maps generated by Dual Systemy Plotter technique
243 (<https://github.com/CJ-Chen/TBtools>). Finally, the result was visualized
244 using TBtools v2.225.

245 ***Cis*-regulatory elements analysis of *OsLOXs***

246 The 2000 bp upstream sequences of each *OsLOX* promoter region were
247 obtained from the Phytozome v13 database [49] to investigate the *cis*-
248 acting regulatory elements (CREs) and their functions. To identify the
249 prospective *cis*-regulatory elements (CREs) within the promoter region of
250 *OsLOX* genes, the PlantCARE database
251 (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [75] was
252 employed and the potential CREs were visualized using TBtools v2.225
253 [63].

254 **Specification putative miRNA targeting *OsLOX* genes**

255 To identify potential miRNAs that target the *LOX* genes in rice,
256 psRNATarget (<https://www.zhaolab.org/psRNATarget>) [76] was utilized by
257 uploading the CDS sequences of *OsLOXs*. The analysis focused on the
258 mature micro-RNAs of rice listed in miRbase (<https://www.mirbase.org/>)
259 [77]. The associations between these miRNAs and their target *OsLOX*
260 genes were illustrated through Cytoscape software [78].

261 **Gene Ontology Analysis**

262 To ascertain the functional relationship of the discovered *OsLOX* genes,
263 gene ontology (GO) enrichment analysis was performed in collaboration
264 the Plant Transcription Factor Database ([https://plantregmap.gao-](https://plantregmap.gao-lab.org/go.php)
265 [lab.org/go.php](https://plantregmap.gao-lab.org/go.php)) [79]. ChiPlot (<https://www.chiplot.online>) [80] was
266 employed for data visualization.

267 **Prediction of phosphorylation sites**

268 For the prediction of phosphorylation sites, the protein sequences were
269 used as input using the NetPhos-3.1 website
270 (<https://services.healthtech.dtu.dk/services/NetPhos-3.1/>) [81]. Results
271 with a combined score greater than 0.5 were reliable.

272 **Protein-protein interaction (PPI) network prediction**

273 STRING version-12.0 (<https://string-db.org/>) [82] was employed to predict
274 the PPI network of *OsLOX* proteins based on the homologous proteins from
275 *Arabidopsis thaliana*. The following parameters of STRING tool were
276 specified for the analysis: (i) full STRING network, as network type, (ii) the
277 meaning of network edges as evidence, (iii) minimum interaction score 0.4
278 (medium confidence parameter), and (iv) maximum number of interactions
279 in first shell <10. Cytoscape software [78] was employed to visualize the
280 predicted interaction network. Further, putative functions of the
281 interacting protein families and result of KEGG enrichment analysis of PPI
282 node proteins were retrieved.

283 **Secondary and tertiary structure analysis of *OsLOX* proteins**

284 The STRIDE program (<https://webclu.bio.wzw.tum.de/stride/>) [83] with
285 default parameters was employed to predict OsLOX protein's secondary
286 structure. PROTEUS Structure Prediction Server 2.0
287 (<http://www.proteus2.ca/proteus2/>) [84] was employed to forecast the
288 turns, coils, 310 helices, extended beta sheets, and alpha helices in the
289 context of structural analysis. Additionally, investigation of membrane-
290 spanning motif was done by using DeepTMHMM 2.0
291 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) [85].

292 Next, the trRosetta webserver (<https://yanglab.qd.sdu.edu.cn/trRosetta/>)
293 [86] were employed for generating the tertiary structure of OsLOX
294 proteins. The predicted tertiary structures were refined using the
295 GalaxyRefine2 module of the GalaxyWEB server
296 (<http://galaxy.seoklab.org/>) [87]. Energy minimization was done employing
297 Swiss-PdbViewer v4.1 (<http://www.expasy.org/spdbv/>) [88]. To verify the
298 reliability of the protein structures, PROCHECK tool
299 (<https://servicesn.mbi.ucla.edu/PROCHECK>) [89] and ERRAT servers
300 ([https:// servicesn.mbi.ucla.edu/ERRAT/](https://servicesn.mbi.ucla.edu/ERRAT/)) [90] were utilized for assessing
301 model quality. In addition, Z-scores and energy plots were assessed using
302 ProSA-web (<https://prosa.services.came.sbg.ac.at/prosa.php>) [91]. Finally,
303 the 3D structures were visualized using the Pymol 3.1 software [92].

304 **Spatiotemporal expression profiling of the genes in rice using RNA-** 305 **seq data**

306 The RGAP database [50] and Rice Expression Database
307 (<https://ngdc.cncb.ac.cn/red/index/>) [93] were employed for collecting

308 RNA-seq expression data of twelve *OsLOX* transcripts in various tissues
309 throughout several development phases. Plant Public RNA-seq Database
310 (<https://plantrnadb.com/ricerna/>) [94] was employed with RGAP Locus IDs
311 of OsLOXs to collect transcriptomic data of rice during different time
312 points for biotic and abiotic stress conditions. To quantify gene expression,
313 sample transcript value was normalized using Log2 transformation
314 method (FPKM + 1) [95] for each gene. GraphPad Prism 10.5.0
315 (<https://www.graphpad.com/>) [96] software was used to visualize a heat
316 map derived from the expression profile data.

317 **Plant germination, abiotic stress treatments, and total RNA** 318 **isolation**

319 Seeds of the *indica* rice variety BRRI Dhan 105 were procured from the
320 Bangladesh Rice Research Institute (BRRI), with only the healthy, mature,
321 and high-quality seeds chosen for this experiment.

322 Thoroughly cleaned seeds were positioned in a petri dish with wet tissue
323 paper to initiate germination. After three to four days, germinated
324 seedlings were transferred into a hydroponic cultivation system. In the
325 growth chamber, environmental conditions were regulated at a
326 temperature of $25 \pm 2^{\circ}\text{C}$, with a photoperiod of 16 hours of light and 8
327 hours of darkness, and a light intensity of 1500–2000 lux. 21 days old
328 seedlings were subjected to several abiotic stress treatments which
329 included salt (150 mM NaCl), drought (3 mM PEG 6000), saline-alkalinity
330 (60 mM NaHCO_3), heat (42°C), cold (4°C) and IAA (98mg/L dissolved in
331 NaOH followed by distilled water) for 24h; and samples were taken at two

time intervals: 12 hours and 24 hours, to ensure optimal responsiveness. The untreated seedlings were employed as a control. Fresh young leaves collected 12h and 24h post-treatment were thoroughly cleaned with 70% ethanol and distilled water for removing any pollutants prior to RNA extraction. Total RNA of treated and controlled frozen leaf tissue samples was extracted following the FavorPrep™ Tri-RNA Reagent user guide's procedure. The removal of genomic DNA contamination was conducted with DNase I from Thermo Fisher Scientific Corporation's Invitrogen™ DNA-free™ DNA Removal Kit. The first-strand cDNA of the mRNA was produced with the ABscript II cDNA First Strand Synthesis Kit (ABclonal, Inc. USA), in accordance with the manufacturer's instructions.

Expression profiling under abiotic stress conditions using quantitative real-time PCR data

The primer design for the RT-qPCR was conducted using the NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) [97] and the OligoAnalyzer Tool (<https://sg.idtdna.com/pages/tools/oligoanalyzer>) of Integrated DNA Technologies, Inc. The product length was maintained between 160 and 220 bp (Table S1). To examine the expression of each of the twelve *OsLOX* genes expression regarding treatments, the RT-qPCR was conducted employing a 96-well plate format on the BioRad CFX96™ Real-Time PCR Detection System. Eukaryotic elongation factor 1 alpha (eEF-1α) [98] was chosen for internal reference. The study utilized the GoTaq® qPCR Master Mix (2X) from Promega Corporation, USA. For each 15 µL reaction mixture, we incorporated 7.5 µL of GoTaq® qPCR Master Mix (2X), 2 µL of 10-fold diluted cDNA, 1 µL of gene-specific primers, and

3.5 μ L of nuclease-free water. Each reaction was conducted under the following conditions: initial denaturation for 2 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds, and extension at 72°C for 40 seconds. The annealing temperature was 63.4°C for *OsLOX1*, 61.6°C for *OsLOX2* and *OsLOX10*; 63.5°C for *OsLOX3*, *OsLOX8* and *OsLOX5*; 62.6°C for *OsLOX4*, *OsLOX9* and *OsLOX11*; 61.1°C for *OsLOX6*, 61.3°C for *OsLOX7* and *OsLOX12*; 59.6°C for *eEF-1 α* . All reactions were performed in triplicate for each experimental condition and melting curve analysis was conducted after PCR amplification. The double delta Ct value method was used to determine the relative gene expression levels [99]. To ascertain the mean values of various treatments, technical replication was implemented and further, data processing and statistical analysis was performed using Microsoft Office 365 and GraphPad Prism 10.5.0 [96]. In each treatment group, the experimental data were represented as the mean $\pm$ standard deviation (SD) of the measured values. GraphPad Prism v10.5.0 was employed to generate bar graphs. A significance test was conducted utilizing two-way ANOVA, succeeded by Dunnett's test. The asterisks (*) above the bars denote statistically significant differences ($p < 0.05$, Dunnett's test) among various time points.

Results

Sequence curation of Lipxygenase genes in *Oryza sativa* genome and physicochemical properties analysis

A cumulative of twelve *LOX* gene family members were identified in the genome of *Oryza sativa*. Six *AtLOX*'s protein sequences were used as a

reference to perform BLASTP searches against the *Oryza sativa* v7_JGI dataset on Phytozome [49]. A total of 24 matches were identified, and of the 24 transcripts analyzed, 17 were confirmed to include both the Lipxygenase domain and the PLAT/LH2 domain through sequence analysis using a Hidden Markov Model (HMM) profile, as these are the defining characteristics of LOX genes. These 17 transcripts correspond to the twelve LOX genes that display alternative splice variants. A BLASTP search was conducted in the RGAP [50] and NCBI protein database [51] to ensure that no potential LOX protein sequence was overlooked. The representative transcripts for the genes in this investigation were selected from the Phytozome annotated primary transcripts. Based on chromosomal number and location, the genes were named sequentially (*OsLOX1* - *OsLOX12*), where 'Os' prefix was used for *Oryza sativa*, followed by *LOX* for *Lipxygenase* [64]. Information regarding the conserved domains positions within the twelve OsLOX proteins is stated in Table S2.

To get a comprehensive idea regarding physicochemical properties of OsLOXs proteins, sequence characterization was done. The proteins length ranged from 347 to 942 aa, along with CDS length ranging from 1041 bp (*OsLOX12*) to 2826 bp (*OsLOX9*). The molecular weights of the deduced OsLOX proteins varied from 39,243.14 Da for *OsLOX12* to 104,687.15 Da for *OsLOX11*. The proteins exhibited an average molecular weight of 92,146.14 Da. Analysis of the instability index revealed that four out of seventeen transcripts had an instability score below 40, indicating their stability. It was determined that 24% of the genes were stable, while

76% were unstable. Isoelectric point (pI) study revealed that eight OsLOX proteins had pI value <7.0, categorizing them as acidic, while four OsLOX-encoded proteins displayed pI values >7.0, signifying their basic characteristics. Furthermore, the negative GRAVY value of all the proteins indicates that the OsLOX proteins were likely hydrophilic. Subcellular localization prediction was conducted to further the understanding of the biological processes and functions of OsLOX proteins within various organelles. This confirmed the predominant presence of the highest quantity of OsLOX proteins in the chloroplast, cytoplasm and mitochondria. Both the vacuole and the plasma membrane contain OsLOX proteins; however, only OsLOX12 was found in the vacuole while OsLOX6 and OsLOX12 were found in the plasma membrane (Figure S1). All the attributes of OsLOX gene family members along with their physicochemical properties are mentioned in Table 1. MSA revealed that all OsLOX genes exhibit significant sequence similarity, possessing two highly conserved domains at their N- and C-termini: the PLAT/LH2 Domain and the Lipxygenase Domain. Position of the characteristic conserved domains of OsLOX proteins is highlighted in Figure 1.

Phylogenetic relationship analysis

In order to investigate the evolutionary relationships among the LOX genes from different species, a phylogenetic tree was generated by aligning the full-length amino acid sequences of the highly homologous LOX protein sequences from: *Oryza sativa*, *Arabidopsis thaliana* [36], *Zea mays* [64], *Brassica rapa*[65], *Sorgum bicolor* [66], *Setaria italica* [40] and *Glycine*

max [37]. An unrooted tree was generated via the Maximum Likelihood approach in MEGA11 [58]. Poisson correlation and pairwise deletion were selected as the mode and gap, respectively, with 1000 bootstrap repetitions employed as the validation parameter. The phylogenetic tree (Figure 2) illustrated that the twelve OsLOX proteins were categorized into two subfamilies: 9-LOXs and 13-LOXs, with the latter further subdivided into Type I and Type II. In detail, the subfamily 9-LOXs contains 4 OsLOX proteins (OsLOX3, OsLOX4, OsLOX5 and OsLOX10) along with 2 AtLOX, 3 BrrLOX, 7 SiLOX, 5 SbLOX, 6 ZmLOX, and 22 GmLOX proteins. Maximum of 7 OsLOX (OsLOX1, OsLOX2, OsLOX6, OsLOX8, OsLOX9, OsLOX11 and OsLOX12) proteins belonged to 13-LOX Type I; this subfamily also contained 4 AtLOX, 12 BrrLOX, 5 SiLOX, 3 SbLOX, 6 ZmLOX, and 12 GmLOX proteins. Only the OsLOX7 protein from the rice genome is present in 13-LOX Type II, which also comprises 2 GmLOX, 1 ZmLOX, 1 SbLOX, and 1 SiLOX proteins. Subfamily 13-LOX Type II solely included monocots, except for one GmLOX which further suggests that these genes were exclusive to monocotyledons[64].

Gene structure and conserved motif analysis

Exon-intron structure is a significant evolutionary characteristic of genes that offers essential insights into functional diversity. The analysis of exon-intron structures (Figure 3a) indicated that the overall number of coding sequences (CDS) or exons varied from 3 to 10, while the number of introns ranged from 2 to 9. *OsLOX10* possessed the lowest number of exons and introns, comprising 3 exons and 2 introns, whereas *OsLOX3* exhibited the

greatest amount, consisting of 10 exons and 9 introns. There was no intron-less gene present. The diversity in the lipoxygenase gene family of rice indicates varied functional roles and evolutionary divergence within this gene family.

For identifying conserved motif distribution and diversity analysis of *OsLOX* genes, web-based tool MEME [70] were employed. 15 conserved motifs were found and designated as motifs 1 through 15 (Figure 3.b). Majority of the motifs were shared by the members due to the strong sequence similarity between *OsLOX* peptide sequences. Among the 15 motifs, motifs 4, 5, 8, 11 and 14 were shared by all the *OsLOX* proteins. Apart from *OsLOX7*, motif 15 was found in the remaining 11 *LOX* proteins. *OsLOX12* is devoid of all other motifs, indicating its uniqueness within subfamily 13-*LOX* Type I. Except *OsLOX6*, remaining motifs were shared by the members of all subfamilies. Functional analysis of the motifs revealed that all the motifs belong to Lipoxygenase iron binding catalytic domain profile (Table S3).

Chromosomal distribution analysis

The investigation of chromosomal mapping analysis of *OsLOX* genes indicated that all *OsLOX*s are distributed randomly and unevenly throughout 7 of the 12 chromosomes in rice (Figure 4). No *LOX* members were mapped onto chromosomes 1, 6, 7, 9, and 10. Chromosome 3 contained 4 *LOX* genes apiece, while chromosomes 8 and 12 each possessed 2 *LOX* genes. A single *LOX* gene was present on each of the following chromosomes: 2, 4, 5, 11. *OsLOX1* and *OsLOX2* are located on

479 the p arm of their respective chromosomes, while the remaining genes are
480 located on the q arm. *OsLOX7* were positioned near the centromere. Table
481 S4 lists the chromosomal locations, positions, and orientations of all the
482 *OsLOX*s.

483 **Gene duplication prediction and collinearity analysis**

484 Gene duplication study aids in understanding the evolution of plants by
485 emergence of novel gene functions and expansion of gene families.
486 Findings revealed the evolutionary links of *OsLOX* genes and the existence
487 of selective pressure, including neutral, positive, and negative/purifying
488 selections (Table 2). Four pairs of duplicated genes were identified from
489 McScanX result. The result revealed that two pairs of genes
490 (*OsLOX3/OsLOX5* & *OsLOX2/OsLOX6*) were segmentally duplicated, while
491 two pairs found in the 100 kb region of same chromosome
492 (*OsLOX8/OsLOX9* & *OsLOX11/OsLOX12*) were tandem duplicated. To
493 estimate the selection pressure: neutral selection was denoted by $Ka/Ks =$
494 1, purifying selection by $Ka/Ks < 1$, and positive selection by $Ka/Ks > 1$
495 [72]. Duplicated pairs Ka/Ks fraction were all constantly remaining below
496 1, indicating a notable presence of strong purifying selection. Findings also
497 showed that duplication events presumably took place between 46.94596
498 and 4.72241 million years ago (MYA), demonstrating the more ancient
499 duplication events have occurred in *OsLOX3/OsLOX5*; in contrast,
500 *OsLOX8/OsLOX9* was the most recent duplicated pair, indicating recent
501 divergence events of tandem duplicated pairs.

Table 2. Calculations of divergence time and Ka/Ks ratios in the duplicated pairs of *OsLOXs*.

Duplicated gene pair	Ka	Ks	Ka/Ks	Time (MYA)	Purifying assortment	Duplicate type
<i>OsLOX3/OsLOX5</i>	0.1776 96	1.4083 79	0.1261 71	46.945 96	Yes	Segmental
<i>OsLOX2/OsLOX6</i>	0.3250 64	1.2425 97	0.2616	41.419 91	Yes	Segmental
<i>OsLOX8/OsLOX9</i>	0.0605 22	0.1416 72	0.4271 95	4.7224 1	Yes	Tandem
<i>OsLOX11/OsLOX12</i>	0.1290 2	0.1856 34	0.6950 22	6.1878 0	Yes	Tandem

Intraspecific collinearity analysis was conducted to elucidate the potential regulatory functions associated with the highly conserved gene order of the *OsLOX* gene family. Circos tool was used to display the linked gene pairs. This study identified 4 collinear gene pairs within rice genome. Chr3 and Chr4 contained two segmentally duplicated pairs while Chr8 and Chr12 contained two tandemly duplicated pairs (Figure 5a). Comparative interspecific collinearity analysis maps were constructed among rice *LOXs* and two other plant species (*Zea mays* as a monocot and *Arabidopsis thaliana* as a dicot species) for studying the potential evolutionary

relationship between identified members of these species. Seven orthologous pairs were identified (Figure 5b, Table 3). The Ka/Ks ratio ranged from 0.363 to 1.345, with a mean of 0.323. However, an exception was found between rice and Arabidopsis due to high sequence divergence value ($pS \geq 0.75$) which led to Ks saturation. Highest quantity of orthologous gene pairs identified between *Zea mays* and rice demonstrated the substantial impact of duplication events in monocot species, leading to the expansion and functional diversity of the *LOX* gene family during evolution.

Table 3. Duplication events involving rice LOX genes and other species.

Duplicated Gene 1	Duplicate Gene 1	Ka	Ks	Ka/Ks	Time (MYA)	Purifying assortment	Duplicate type
<i>OsLOX2</i>	<i>AT1G72520.1</i> (<i>A.thaliana</i>)	0.2799	$pS \geq 0.75$	N/A	N/A	Possible	Segmental
<i>OsLOX1</i>	<i>Zm00001eb206040_T001</i> (<i>Zea mays</i>)	0.1899	0.3632	0.523	12.106	Yes	Segmental

<i>OsLOX2</i>	<i>Zm00001e</i> <i>b005920_</i> <i>T003</i>	0.06 94	0.49 96	0.13 9	16.65 4	Yes	Tandem
<i>OsLOX3</i>	<i>Zm00001e</i> <i>b216870_</i> <i>T004</i>	0.26 13	1.34 59	0.19 42	44.86 2	Yes	Segmen tal
<i>OsLOX6</i>	<i>Zm00001e</i> <i>b423430_</i> <i>T001</i>	0.15 35	0.43 9	0.34 97	14.63 4	Yes	Segmen tal
<i>OsLOX6</i>	<i>Zm00001e</i> <i>b081610_</i> <i>T002</i>	0.14 6	0.41 35	0.35 32	13.78 2	Yes	Segmen tal
<i>OsLOX8</i>	<i>Zm00001e</i> <i>b035010_</i> <i>T001</i>	0.18 4	0.41 4	0.44 44	13.80 1	Yes	Tandem

523 ***Cis*-regulatory elements (CREs) analysis of *OsLOXs***

524 To investigate the potential regulatory mechanisms of *OsLOXs* in the
525 presence of a variety of stimuli and to comprehend the pivotal role they
526 play in transcriptional regulation and gene expression, the CREs of the
527 promoter region of each *OsLOXs* (up to 2.0 kb upstream of the translation
528 start site) were searched against PlantCARE database [100]. There were
529 46 *cis*-regulatory elements found within the 5' UTR promoter region of
530 *OsLOXs* and grouped into seven different functional categories, namely
531 elements responsible for i) Light (14.5% of all identified CREs), ii) Abiotic

challenges (4.6%), iii) Hormonal regulation (12%), iv) Cellular development (2%), v) Promoter associated (63.7%), vi) Biotic challenges (2.2%), and vii) Miscellaneous functions (1%). The aspects of hormonal regulation were categorized into a) Methyl jasmonate (MeJA), b) Salicylic acid, c) Auxin, d) Gibberellin, and e) Absciscic acid responsive elements. The entire list of identified CREs along with their respective functions was included in Table S5. The prevalence of presumed CREs within the promoter region of *OsLOXs* were depicted in Figure 6.

Various abiotic stress related elements are involved in anaerobic, anoxic, cold, drought and defense responses. The abiotic stress responsive *cis*-elements identified in the promoter region of *OsLOXs* include ARE, TC-rich repeats, MBS, LTR and GC-motifs. Furthermore, biotic stress responsive *cis*-elements including the W box, WUN-motif, and WRE3 were implicated in responses to fungal elicitors and wounding. Additionally, all *OsLOX* genes were characterized by the presence of core promoter elements TATA box and CAAT box. They predominantly operate within the promoter and enhancer regions. Hormonal responsive CREs include ABRE, TGA-element, ARE-motif, P-box, AuxRR-core, TATC-box, TGACG-motif, CGTCA-motif and TCA-element, which were essential for five distinct hormones. The seed specific regulation, zein metabolism, circadian control, meristem and endosperm expression were all dependent on cellular development responsive CREs including O2-site, CAT-box, GCN4-motif, RY-element, and Circadian. Light responsive elements include Box 4, Gap-box, G-box, AE-box, I-box, TCCC-motif, AT1-motif, GT1-motif, ATCT-motif, Sp1, LS7, GA-motif, TCT-motif, GATA-motif, ACE, LAMP-element, GTGGC-motif, and

MRE. In addition to these, CCAAT-box, A-box, HD-Zip 3, and AT-rich element exemplify cis-elements with diverse roles. The notable abundance of CREs within the promoter region of *OsLOXs* indicates their involvement in the transcriptional regulation. Furthermore, a variety of hormones and stress-related CREs point to a direct relationship between the gene's activity in rice under various stress conditions. To completely understand the intricate interactions between CREs of the *OsLOX* gene family and the related signaling pathways in controlling rice stress responses, further research is required.

Specification putative miRNA targeting *OsLOXs*

MicroRNAs (miRNAs) are essential in both plants and animals as post-transcriptional regulators of gene expression. miRNAs downregulation signifies an increased expression of the targeted mRNA. The psRNATarget database [76] was employed for identifying miRNAs targeting the *LOX* genes in rice. The study identified 151 potential candidate miRNAs with a length of 20-24 nucleotides that target *OsLOXs*. The lowest quantity of miRNAs (1) targeted *OsLOX12*, while the maximum quantity of miRNAs (25) targeted *OsLOX6*. The total count of miRNAs that targeted *OsLOXs* was as follows: 19 for *OsLOX1*, 4 for *OsLOX2*, 14 for *OsLOX3*, 17 for *OsLOX4*, 10 for *OsLOX5*, 25 for *OsLOX6*, 20 for *OsLOX7*, 12 for *OsLOX8*, 15 for *OsLOX9*, 6 for *OsLOX10*, 8 for *OsLOX11*, and 1 for *OsLOX12*. The regulatory interactions between putative miRNAs and their targeted *LOXs* are illustrated in Figure 7. Among all the miRNAs, 25 targeted several *OsLOXs*, while the rest miRNAs were exclusive to each gene (Table S6). The majority of miRNA-mediated *OsLOX* repression was accomplished by

mRNA cleavage, with only a small percentage of targets experiencing inhibition during the translation phase. Consequently, this demonstrates that the discovered miRNAs might have the potential to post-transcriptionally influence the production of *OsLOXs* via cleaving mRNA and suppressing translation.

Gene Ontology Analysis

Gene ontology enrichment analysis was conducted to determine a variety of regulatory activities of *OsLOX* genes. It establishes a framework classifying genes into three distinct groups based on their biological processes, cellular components and molecular functions (Figure 8). It has been estimated that there are 22 GO terms across all *OsLOXs*. Among the three groups, the biological process was the most frequent as it contains 16 GO IDs. However, there were three GO IDs in each of the classes of molecular functions and cellular components. Moreover, the functional analysis involving the corresponding GO terms for biological process regions identified that GO terms responsible for the following processes: the biosynthesis and metabolism of oxylipin; the oxidation and modification of lipids; the biosynthesis and metabolism of fatty acids; the biosynthesis and metabolism of carboxylic acids; the biosynthesis and metabolism of organic acids; and the metabolism of oxoacids. GO terms associated with molecular processes found out they are responsible for oxidoreductase and dioxygenase activity, which are characteristic features of *LOX* genes across diverse species. Cellular components associated with these GO terms are located within the cytoplasm, plastid, and chloroplast.

GO analysis regarding the predicted *OsLOX* genes with their respective p-values are listed in Table S7.

Phosphorylation sites prediction

The NetPhos 3.1 service predicted the phosphorylation sites for serine (Ser), threonine (Thr), and tyrosine (Tyr) of eukaryotic proteins through neural network integration. The *OsLOX* protein family possesses multiple unique phosphorylation sites throughout its amino acid sequences, amounting to a total of 1473 phosphorylation sites. In particular, 778 of the total phosphorylation sites were made up of serine residues, with threonine and tyrosine accounting for 552 and 143 residues, respectively. The most serine phosphorylation site was *OsLOX8* with 114, while the least number was *OsLOX12* with 35. The most likely phosphorylation site had a value of 0.998 (much larger than the threshold value of 0.500) [101], which indicates predominant kinase specificity; the most threonine phosphorylation sites was *OsLOX6* with 73, and the least abundant was *OsLOX12* with 24, of which the most likely phosphorylation site had a value of 0.994; the most abundant tyrosine phosphorylation site was *OsLOX7* with 20, while the least abundant was *OsLOX9* with only 4, of which the most likely phosphorylation site had a value of 0.988. It is suggested that the *OsLOXs* functional variety is determined by proteins with distinct architectures and kinase-specific phosphorylation sites (Figure 9).

Protein-protein interaction (PPI) network prediction

Analysis of PPI network demonstrated the possible regulatory roles of *OsLOX* proteins based on the strong homology of orthologous *Arabidopsis*

STRING proteins. Apart from OsLOX11, the family was found to be homologous with 10 *Arabidopsis* proteins (CYP74A1, CYP74A2, CYP74A3, CYP74A4, SLM1, AOC, Q2RAM0_ORYSJ, Q2R2W3_ORYSJ, PLA2-II, and PLA2-III). Additionally, KEGG enrichment analysis indicated that proteins that interact with *OsLOXs* may have the potential to participate in hormone-mediated pathways and external stress responses (Figure 10, Table S8).

Secondary and tertiary structure analysis of OsLOX proteins

Secondary structural analysis of OsLOXs revealed the percentages and locations of different constituents; as well as the location of transmembrane helix (Table S9). With exception of OsLOX1, the predominant secondary structure was the alpha-helix, closely succeeded by the turn, coil, beta-sheet, and 310-helix. OsLOX1 has the highest turn in its structure which indicates a compact and flexible protein structure, it might be involved in binding or signaling processes. Moreover, the stability of OsLOXs is suggested by the higher proportion of helical structures [102], and structures such as random coils are essential for signaling cascades [103]. Only OsLOX6 had single membrane spanning motif (MSM) at 664 to 673bp position (Figure 11).

The trRosetta webserver [86] is a deep learning algorithm that employs a neural network for predicting inter-residue geometry (orientations and distances) and builds tertiary models using RosettaTTAfold. GalaxyRefine [87] further refined the generated models, and then Swiss PdbViewer [88] was utilized for energy minimization. Figure 12 depicts the predicted 3D

structures of OsLOX proteins, generated using Pymol 3.1 Software [92]. Multiple validation tools were implemented to assess the structural reliability of predicted protein models (S10 table). Ramachandran plot analysis through PROCHECK [89] revealed that more than 90% of the residues were situated within the most favorable and additionally allowed regions, while fewer than 1.5% were positioned in disallowed regions, indicating a high-quality stereochemical conformation (Figure S2). ERRAT analysis [90] further supported this with models exhibiting an overall quality factor exceeding 87, suggesting minimal structural errors (Figure S3). ProSA-web analysis [91] yielded Z-scores that are within the typically observed range for experimentally determined native proteins and serve as evidence for the reliability of the predicted structures (Figure S4). The corresponding energy plots confirmed that all residues were associated with favorable (low) energy values, indicative of structurally stable regions (Figure S5). The validation results collectively affirm the high quality and dependability of the produced tertiary protein models.

Spatiotemporal expression profiling of the genes in rice using RNA-seq data

Gene expression analysis measures the functional activity and expression level of active gene products (functional RNA or protein) in different cells and tissues. This is a highly specific molecular indicator of biological activity and metabolite levels. Changes associated with gene expression patterns are linked to corresponding alteration in development, disease progression, and adaptation to external stresses. In this study, RNA sequencing data of 12 *OsLOXs* in different tissues were retrieved from

679 RGAP database [50] and Rice Expression Database [93] to investigate
680 spatiotemporal effects during developmental stages for corresponding
681 genes. The generated heatmap visualized the RNA transcript profiles with
682 normalized expression values of RNA-seq FPKM (Figure 13).

683 The analysis identified low to moderate expression levels in most tissues,
684 with several members exhibiting strong tissue-specific expression. For
685 instance, *OsLOX1* displayed high level of expression in leaf (7 DBH to 7
686 days DAF) and shoot (7-day seedling and 14 DAG), this indicates its
687 potential role in leaf development. *OsLOX8* and *OsLOX9* were upregulated
688 in anthers during flowering stage indicating its role in pollen maturation.
689 In the same manner, *OsLOX4* and *OsLOX6* were moderately upregulated
690 in roots which implies potential participatory role in root growth or
691 defense signaling. *OsLOX2* was upregulated in anther and glumes, while
692 *OsLOX5* exhibited a mild upregulation in root (14 DS and 21 DAS). *OsLOX4*
693 showed notable upregulation during early germination (8 h after
694 imbibition), whereas other members were downregulated in germinating
695 seed and mature seed tissues. *OsLOX6* showed overall moderate
696 upregulatory expression for most tissues. *OsLOX3*, *10*, *11*, and *12*
697 expression level were downregulated for all tissues.

698 To get a better understanding of how *OsLOXs* respond to various stresses,
699 the expression patterns of *OsLOXs* were examined in response to various
700 biotic and abiotic stressors. The Plant Public RNA-seq Database [94] was
701 employed to acquire RNA-seq data for the investigation. GraphPad Prism
702 10.5.0 [96] was used to generate the corresponding heatmaps. Log2 values

of relative expression are represented by the color gradient on the right side of the heatmap (Figure 14). In response to infection by bacterial leaf streak pathogen (*Xanthomonas oryzae* pv. *Oryzae*), *OsLOX1*, 2, 4, 5, 6, 7, 8, and 9 were notably upregulated, while *OsLOX3*, 10, 11 and 12 displayed downregulation. In the event of infection by the rice blast fungus (*Magnaporthe oryzae*), *OsLOX1*, 2, 4, 6, 7, 8, 9, and 11 exhibited consistent upregulation from 8 h to 72 h post-inoculation, suggesting their active participation in defense signaling against fungal invasion. Remaining genes were downregulated. Additionally, infection by rice stripe virus (RSV) led the upregulation of *OsLOX1*, 2, 4, 6, 7, and 11 at 3 to 15 days post-inoculation (DPI). *OsLOX8* was upregulated in 3 DPI, but prolonged infection led to suppression of the gene. Infection by necrotrophic fungus *Rhizoctonia solani* (Sheath blight) resulted in upregulation of *OsLOX2*, 6, 7, and 11 at 1 to 3 DPI, *OsLOX8* and 9 showed delayed upregulation (3 DPI); in contrast *OsLOX3*, 5, 10 and 12 were markedly downregulated. Rice black streaked dwarf virus (RBSDV) infection triggered a high upregulation of *OsLOX1*, 2, 4, 7, and 11, indicating a possible role in antiviral defense.

Pyricularia oryzae is another fungal pathogen responsible for rice blast disease, exhibited upregulation of all genes of OsLOX family apart from *OsLOX3*, 5, 10, and 12. Similarly, fungal blast causing *Magnaporthe grisea* increased the expression of *OsLOX2*, 3, 4, 5, 6, and 7. Moreover, *Meloidogyne graminicola* (root knot nematode) infection induced the expression of *OsLOX1*, 2, 4, 7, 8, 9, and 11 at both 3 and 7 days after infection (DAI). Striga infection also led to upregulation of *OsLOX4*, 5, and

728 6 at infection stage of 3 and 7 days, while *OsLOX1*, 10, 11, and 12 remained
729 significantly downregulated. *Xylaria striata* is an endophyte that exhibits
730 improved disease resistance by upregulating *OsLOX4*, 5 and 9. Plant
731 infected with rice root nematode (RRN) *Hirschmanniella* showed
732 upregulation of *OsLOX3*, 6, and 9. In conclusion, these results address
733 potential role of *OsLOXs* in rice defense mechanisms against pathogens
734 and biotic stress conditions.

735 Treatment with abiotic stress caused a noticeable change in *OsLOXs* gene
736 expression patterns over a range of time periods. In response to
737 submergence stress, *OsLOX2*, 4, 8, 9 and 11 were upregulated consistently
738 at all time points, suggesting a potential role in submergence adaptation
739 (Figure 15). The remaining genes were consistently downregulated.
740 During drought stress, *OsLOX4* and 11 were consistently upregulated
741 under 1 to 24 hours of exposure. *OsLOX1* and 2 showed upregulation
742 during first 3 hours of treatment, *OsLOX8*, 9, and 12 showed upregulation
743 during 3 to 12 hours of treatment. *OsLOX3*, 5, 6, 7, and 10 were
744 predominantly downregulated, indicating their suppression under
745 dehydration conditions. Similar distinct patterns were observed under
746 saline conditions. *OsLOX1* and 2 showed highly upregulated expression
747 across 1 to 24 hours of ionic imbalance. Short-term salt exposure (1-5 h)
748 induced moderate upregulation of *OsLOX4*, 7, and 11, *OsLOX5* were
749 upregulated during the 2nd hour of treatment, but continued exposure to
750 salt led to downregulation. *OsLOX9* were moderately expressed during 5
751 to 10h of salinity, whereas *OsLOX3*, 6, 10 and 12 were consistently
752 downregulated. In the case of saline alkalinity, *OsLOX2*, 4, 6 and *OsLOX9*

remained highly upregulated, suggesting sensitivity to alkaline ion toxicity. Cold treatment at 4°C resulted in a strong and constant upregulation of *OsLOX4*, *8*, *9*, and *11* from 1 to 168 hours, indicating a possible role in cold acclimation. *OsLOX1* and *7* started with high induction (upregulatory expression) from 0 to 24 hours, but further cold treatment led to downregulation of the transcripts. With the remaining genes showing downregulation across all time points suggest cold mediated transcriptional repression. Heat stress caused constant upregulation of *OsLOX1* and *2* from 30 to 240 mins, whereas *OsLOX 4* and *7* were moderately upregulated, indicating heat-responsive activation. Rest of the genes were downregulated during thermal treatment. These differential expression patterns indicate *OsLOX* family members participate actively in regulating rice adaptation to environmental stressors.

In addition to numerous abiotic treatments, several hormones including auxin, gibberelins, abscisic acid, jasmonic acid, salicylic acid, kinetins, and others aid plants during growth and development. During the biosynthetic process, LOX genes produce JA. Therefore, understanding transcript expression patterns under hormone treatment will aid in our comprehension of the optimal conditions for plant growth. In response to JA treatment, *OsLOX2*, *OsLOX8*, and *OsLOX11* were consistently upregulated at 6, 12, and 24 hours in shoots, highlighting their involvement in JA-mediated signaling. *OsLOX4* and *9* showed moderate expression patterns whereas remaining genes were mainly downregulated across JA time points. During ABA treatment, *OsLOX4*, *8* and *9* were upregulated, with the rest being in downregulation implies that ABA does

not activate these isoforms. In response to auxin treatment, *OsLOX3*, 4, 5, 6 and 8 demonstrated high upregulation in root tissue for weekly treatment. Overall, the *OsLOX* gene family displayed distinct and stress-specific transcriptional responses which implies functional divergence for adverse environmental conditions.

Expression profiling under abiotic stress conditions using quantitative real-time PCR data

The relative expression patterns of *OsLOXs* were assessed in rice seedlings leaves throughout two different time periods to explore the effects of drought, salinity, saline-alkalinity, heat, cold and IAA (indole-3-acetic acid) stress conditions. Figure 16 illustrates a bar chart representing their real-time expression data under various stress situations after 12 and 24 hours.

As shown in Figure 16, *OsLOX1* transcript was significantly upregulated under drought, heat and salt stress and downregulated expression was observed for saline-alkalinity and IAA treatment at 12h. Response to drought and salt stress showed a contrasting downregulatory expression at 24, whereas response persists for saline-alkalinity and IAA treatment. Heat stress response alleviated after prolonged exposure. Cold treatment resulted in no change in transcript expression pattern compared to control. *OsLOX2*, drought and cold response were significantly upregulated at both time points, with cold response demonstrating seven-fold upregulation at 24h. Salinity induced significant upregulation after 24h, while saline-alkalinity showed decrease to increase expression pattern. Heat and IAA treatment resulted in higher upregulation at 12h

802 but *OsLOX2* transcript displayed no significant result at 24h. *OsLOX3*
803 exhibited substantial upregulation of transcript during drought and cold
804 stress. Drought stress caused approximately four-fold significant higher
805 expression at both times, but cold stress issued approximately seven-fold
806 increase of relative expression after 24h. Salt, saline-alkalinity, and heat
807 resulted in significant downregulation under these stressful situations and
808 the downregulation sustained. IAA treatment induced downregulation at
809 12h but further exposure led to no relative change of gene activity.
810 *OsLOX4*, *5*, *6*, and *8* showed similar expression patterns like *OsLOX3*. For
811 *OsLOX5*, drought stress instigated significant upregulation of gene activity
812 from two-fold to approximately four-fold; and cold stress resulting in less
813 than one-fold to more than six-fold transcriptional upregulatory activity.
814 The remaining stress significantly downregulated the transcript, and the
815 downregulation was continuous. *OsLOX5* transcript under drought
816 conditions affected a significant upregulation from more than two-fold to
817 approx. five-fold. Although, there was upregulation of cold-treated
818 transcript at 12h, it was not significant until 24 where it showed approx.
819 three-fold upregulation of relative expression. Like previously stated
820 transcript, salt, saline-alkalinity, and heat treatment showed significant
821 downregulation at both time points. IAA treatment resulted in significant
822 downregulation at only 12h. *OsLOX6* exhibited significantly higher level
823 of expression change for drought conditions, the relative expression value
824 fluctuated from more than three-fold to slightly higher than one-fold. Cold
825 stress elevated the expression at 24h. Remaining stress resulted in
826 significant downregulation, and it persisted till 24h. *OsLOX8* expression

was significantly enhanced under drought condition for both sampling time; under cold condition expression increased at 12h but it became significant only at 24h. Other stress followed the significant downregulatory pattern.

A change for salt-induced stress was discovered in *OsLOX7*, it showed significantly higher levels of expression at 12h; but contrasting effect was observed during 24h with significant downregulation of the transcript. Drought caused significant upregulation of gene activity at 12h (with approx. three-fold change), which slightly decreased at 24h but remained significant. Cold condition caused significant upregulation at 12, IAA depicted significant upregulation at 24h with delayed response. Significant downregulation was observed for heat treatment at 12h and for saline-alkaline treatment at 24h. Regarding the transcript profile of *OsLOX9*, it showed significant upregulation under drought and cold conditions. Heat treatment demonstrated significant downregulation at both time periods; significant downregulation was noticed in salt and saline-alkaline treated samples of 24h. Exception of IAA treatment with notable upregulation was observed at 24h. Moreover, a decrease to increase expression pattern was observed in *OsLOX10* with a significantly higher expression level of over fourfold under cold stress. Rest of the stress including drought situation was significantly downregulated for this transcript. *OsLOX11* had considerably significant upregulation levels of approx. eight-fold during heat (only 24h) and cold (both period) stress conditions. Salt and saline-alkalinity showed significant increase to decrease pattern of expression. Drought and IAA induced significant

upregulation at only 12h. No significant changes were observed in *OsLOX12* transcript for drought, cold and IAA stress situations. Salt, saline-alkalinity resulted in significant downregulation after prolonged period. Heat stress exhibited consistently significant downregulation.

However, under salt and cold conditions the RNA-seq data presented in Figure 15 did not exhibit consistent findings with RT-qPCR data for all gene transcripts. Drought, saline-alkalinity and heat stress induced expression of RT-qPCR relative expression was more or less consistent with RNA-seq data. Thus, these findings show the greatest time dependent differential expression pattern of *OsLOXs*, suggesting its significant role in the adaptation to abiotic stress in rice.

Discussion

Lipoxygenase is a significant dioxygenase encoding protein family that catalyzes the regio and stereoselective dioxygenation of polyunsaturated fatty acids (PUFAs) and serves as precursors for oxylipins. It actively participates in various growth and development phases and enhances resistance to harsh external environmental conditions across diverse plant genotypes [104]. This present study identified 12 distinct *OsLOX* genes in rice whereas fourteen genes had been reported previously [105]. Identified genes possess both characteristic domains, the PLAT/LH2 and Lipoxygenase domains at their respective N and C termini which is consistent with previously identified true *LOX* family members [37,106]. Compared to C3 and C4 plants, *Oryza sativa* has more numbers of gene than in Arabidopsis (six) (Umate, 2011), Sesame (7) [107], Radish (11)

[39], and fewer than Maize (13) [108], Wheat (44) [57], and Cotton (64) [109]. These differences in the quantity of *LOX*s are not proportionate with genome size, suggesting that the *LOX* gene has not been conserved throughout the evolutionary process. Physicochemical analysis revealed these genes differ in sequence length, molecular weight, isoelectric point, and GRAVY score, highlighting the heterogeneity of the gene family. The negative GRAVY score of the proteins verifies their hydrophilic nature [110]. The subcellular localization of the proteins indicated their presence in the chloroplast, cytoplasm, mitochondria, nucleus, and plasma membrane. The localization discrepancies of the proteins predicted by the two independent tools. According to the findings of CELLO v.2.5 [62], most proteins are found in the cytoplasm, whereas WoLF PSORT [61] indicated their localization in chloroplast, mitochondria and vacuolar membrane. Additional verification of protein subcellular localization necessitates validation by a GFP-fused transient expression system employing an Agro-infiltration method [111]. Hence, it could be said that different *LOX* gene members serve catalytic functions in different organelles.

In a multispecies phylogenetic tree constructed with both monocot and dicot species, genes in a subgroup often have similar functions. OsLOX proteins were categorized into two distinct subfamilies: 9-LOXs and 13-LOXs, with the latter further subdivided into Type I and Type II, consistent with Wheat [57] and *Artemisia annua* [112]. However, *LOX* genes in sorghum [66] and pepper [23] were categorized into 9-LOX, 13-LOX and an unknown subfamily [113]. Seven *OsLOX* genes were present in Type I 13-LOXs, but one *OsLOX* gene was present in Type II 13-LOXs with no

901 *AtLOX* sequence belonging to this group; it indicates certain divergences
 902 between *LOX* gene families of monocots and dicots. The structural
 903 divergence of introns and exons in plant species is crucial for evolution
 904 [114]. All *OsLOX* gene family members were identified to have exons
 905 ranging from 3-10, and the intron number ranged from 2-9. Identification
 906 is consistent with the pattern of *Arabidopsis thaliana* [36] and *Brassica*
 907 *rapa* [28]. There was no intron-less gene which indicates that they are
 908 highly expressed and have not undergone recent evolution. Analysis of
 909 conserved motifs revealed that the substantial sequence similarity among
 910 *OsLOX* proteins resulted in shared motifs across all three phylogenetic
 911 categories. Except for *OsLOX6* and *OsLOX12*, all members of Type I 13-
 912 *LOX* exhibited 15 motifs; the absence of a total of 5 and 9 motifs in these
 913 two genes, respectively, indicates their uniqueness within Type I 13-*LOXs*.
 914 The analysis of chromosomal distribution found that all *OsLOX* genes are
 915 dispersed randomly and unevenly throughout 7 of the 12 chromosomes.
 916 No *OsLOX* member was mapped onto chromosomes 1, 6, 7, 9, and 10.
 917 Previous study identified five tandemly duplicated pairs in tomato [115].
 918 Three tandem and three segmental duplicated gene pairs were observed
 919 in *LOX* gene family of poplar [106]. Our research revealed that of the four
 920 duplicated gene pairs, two were tandem duplicates and two were
 921 segmental duplicates. It is possible to assert that the expansion of the *LOX*
 922 gene family in the rice genome is being driven by both segmental and
 923 tandem duplicated gene clusters. Seven orthologous gene pairings were
 924 found among rice, *Arabidopsis thaliana* and *Zea mays*. The observed cases
 925 of gene duplication across various chromosomes indicate segmental

duplication, which serves as the principal driver for diversification. The evolution of the duplicated *LOX* pairs was influenced by purifying selection, as evidenced by Ka/Ks values < 1 [72].

A total of 46 categories of cis-regulatory elements were identified and categorized into seven functional groups through further investigation of the promoter region of the *OsLOX* gene family. These elements include those for cellular development (ABRE, AuxRR-core, ARE-motif, P-box, TATC-box, etc.), light response (CAT-box, Box 4, Sp1, Gap-box, MRE etc.), phytohormones (ABRE, TGA-element, AuxRR-core, P-box, TATC-box, etc.), biotic stress (W box, WUN-motif and WRE3), abiotic stress (ARE, TC-rich repeats, GC-motif, MBS and LTR, etc.) and Promoter associated (TATA box and CAAT box). The most prevalent CREs were associated with promoters, where multiple transcription factors bind and it indicates the complex regulatory mechanisms *OsLOX*s. Our results are consistent with previous research that has emphasized the multifaceted role of *LOX* genes in the stress response [25,38,66,109]. miRNAs are critical regulators of gene expression that are essential for the regulation of plant responses to abiotic and biotic stresses in cross species [116]. A total of 151 unique candidate miRNAs targeting members of the rice *OsLOX* gene family were identified. 25 miRNAs targeted several *OsLOX*s, while the other miRNAs were restricted to individual genes. The majority of miRNA-mediated *OsLOX* silencing resulted in mRNA cleavage, whereas a minor proportion of targets experienced translational inhibition and subsequent transcript degradation, thereby suggesting post-transcriptional regulation and translation inhibition.

951 According to results of gene ontology prediction, the OsLOX proteins of
952 rice function in response to oxylipin, lipid, carboxylic acid, fatty acid and
953 diverse metabolic processes, in addition to participating in organic acid
954 and biosynthetic processes alongside several partners. Furthermore, these
955 findings indicate that oxidoreductase and dioxygenase activities are
956 significant functions of *OsLOX* genes, as revealed by GO analysis
957 regarding molecular function, largely occurring within plant cells,
958 specifically in the plastid, chloroplast, and cytoplasm. Protein-protein
959 interactions are essential for numerous intracellular and extracellular
960 processes [117]. Multiple *Arabidopsis* proteins interact with the OsLOX
961 proteins. Among these, *CYP74A1* to *CYP74A4* functions as Allene oxide
962 synthase 4 which is a growth regulator and signaling molecules in plant
963 defense. *PLA2-II*, *PLA2-III*, *Q2R2W3_ORYSJ* acts as phospholipase or its
964 homologs. *AOC* interacts with auxin-mediated signaling pathway &
965 implicated in NaCl stress response. *OsLOX* genes are abundantly
966 phosphorylated at serine, threonine and tyrosine residues. Identifying the
967 putative phosphorylation sites of proteins helps in understanding of the
968 signal transduction, which is essential for plant development and
969 adaptability to environmental stress [118].

970 Analysis of secondary structures showed that except OsLOX1, alpha helix
971 is the most prevalent secondary structure which surpasses others in
972 dominance. It ensures the stable conformation of proteins, like our
973 findings [102]. Only OsLOX6 was identified to possess a singular
974 membrane-spanning motif (MSM). The tertiary structures of the OsLOX

975 proteins were predicted using several techniques, which validated the high
976 quality of all structure predictions and might be used in further research.

977 The information obtained from the RNA-seq database across various rice
978 tissues was utilized to elucidate the functional roles of *OsLOXs* during
979 growth and developmental phases. *OsLOXs* were discovered as differently
980 expressed using tissue-specific expression pattern analysis. The analysis
981 showed that among the tissues studied, *OsLOX1* exhibited higher
982 expression in leaf (7 DBH to 7 DAH), shoot (7-day seedling) and shoot (14-
983 day seedling). Anther and Anther (flowering) stage showed upregulation
984 of *OsLOX2*, *8* and *9* which might indicate the specific role on reproduction.
985 *OsLOX5* demonstrated mild regulatory pattern in root (14 DS and 21 DAS)
986 whereas, *OsLOX4* were significantly upregulated during early germination
987 stages (8 h after imbibition). Expression levels were moderately
988 upregulated for *OsLOX6* for most tissues. All other genes (*OsLOX3*, *10*, *11*,
989 and *12*) exhibited lowest expression for due tissues. These findings are
990 consistent with predominant expression of *BnaLOX2* in
991 stamens, indicating reproductive function [119]. Proteome profiling of
992 *LOX* gene showed expression in mature seeds, seedling stage, early
993 germination in Arabidopsis, tomato, soybean and cucumber [120-124].

994 Additionally, RNA-seq data was processed to examine the role of *OsLOXs*
995 under various biotic stress conditions. Our analysis demonstrated how
996 *OsLOXs* response to diverse biotic factors including viral, bacterial, and
997 fungal infections, as well as nematode inoculation. When induced with
998 *Xanthomonas oryzae* pv. *oryzae* infection, strong upregulation of *OsLOX1*,

999 2, 4, 5, 6, 7, 8, and 9 were shown and this activates JA-related or oxylipin
 1000 pathways typical of antibacterial responses. Fungal infections caused by
 1001 *Magnaporthe oryzae*, which is rice blast responsible organisms, also
 1002 triggered significant upregulation of several *OsLOXs* (*OsLOX1*, 2, 4, 6, 7,
 1003 8, 9, and 11). Necrotrophic fungal infection caused by *Rhizoctonia solani*
 1004 induces sheath blight in rice and elevated the expression of *OsLOX2*, 6, 7,
 1005 and 11. Fungus *Pyricularia oryzae* is
 1006 also responsible for rice blast disease, and it showed higher activity of all
 1007 genes of *OsLOX* family apart from *OsLOX3*, 5, 10, and 12. Viral infections
 1008 (RSV and RBSDV) led to upregulation of *OsLOX1*, 2, 4, 6, 7, and 11,
 1009 highlighting a possible conserved antiviral role. Root-associated
 1010 pathogens further emphasized context specific responses: nematode
 1011 *Meloidogyne graminicola* upregulated the expression of *OsLOX1*, 2, 4, 7,
 1012 8, 9, and 11, while the beneficial endophyte *Xylaria striata* enhanced
 1013 expression of *OsLOX4*, 5 and 9. Taken together, these patterns address
 1014 dynamic and distinct role of *OsLOXs* depending on pathogen type,
 1015 supporting their central role in coordinating defense mechanisms under
 1016 biotic stressors. This present finding is consistent with previous studies
 1017 where *Arabidopsis thaliana AtLOX* proteins showed defence mechanism
 1018 against bacteria and pathogens via oxylipins through lateral root
 1019 development [18]. *AtLOX2* and *AtLOX3* mutants were shown to control
 1020 early-stage plant nematode infections [125]. *ZmLOX3* of maize acts as a
 1021 root-specific suppressor of primary defence signaling pathways and
 1022 provides resistance against nematodes [126]. In papaya, the

1023 hydroperoxides generated by 13-LOX exhibited significant antifungal
1024 activity against blight disease [127].

1025 Furthermore, the expression profile of *OsLOXs* was examined in the
1026 context of drought, salinity, saline-alkalinity, heat, cold and IAA (indole-3-
1027 acetic acid) in two different time points and the data of RT-qPCR analysis
1028 was compared with RNA-seq to ascertain the role under different abiotic
1029 stress conditions. The expression profiles of the majority of genes were
1030 broadly similar between the RNA-Seq and RT-qPCR results, despite the
1031 minor deviations observed during salt and cold stress. In RT-qPCR
1032 analysis, certain reference or housekeeping genes may occasionally
1033 exhibit significant responses to changes in experimental conditions or
1034 tissue types [128,129]. In our study, we used *eEF-1α* to validate the qPCR
1035 result for gene expression [98].

1036 Because of drought and cold stress, *OsLOX* genes showed tolerance at
1037 different time points with upregulated transcripts. *OsLOX* genes were
1038 identified as pivotal in modulating responses to drought and cold
1039 conditions. Under drought stress, *OsLOX1-9* and *OsLOX11* genes showed
1040 significant upregulation, which indicates their possible involvement in
1041 drought tolerance mechanisms. In contrast, *OsLOX10* and *12* were
1042 downregulated or showed no significant change under drought conditions,
1043 indicating gene-specific divergence within the *LOX* family. Under cold
1044 stress, several transcripts exhibited significant and sustained
1045 upregulation. *OsLOX2-11* had significantly higher expression levels at
1046 almost both sampling periods, which aid plants in surviving cold

environment. In contrast, *OsLOX1* and *12* did not exhibit significant changes under cold treatment. Expression was predominantly suppressive in nature under saline condition. *OsLOX3-6, 8-10* and *12* showed significant downregulation, whereas *OsLOX1, 2, 7* and *11* displayed time varying upregulation pattern. Under saline-alkalinity, with exception of *OsLOX2* and *11*, all genes exhibited significant downregulation. Except for *OsLOX1, 2* and *11*, heat stress reported downregulation for *OsLOX* gene family, which suggest suppression of non-essential processes and dominating stress responsive pathways. For IAA treatment, *OsLOX1, 3-8*, and *10-11* significantly downregulated. *OsLOX2* and *9* showed upregulation and *OsLOX12* exhibited no significant changes across treatments. Similar studies have shown upregulatory expression pattern due to drought stress in *Amorpha fruticosa* L. (*AfLOX4*) and downregulation due to saline alkaline conditions, which is persistent with our study [130]. Several genes of maize exhibited transient expression patterns during cold stress, presumably signifying a delayed involvement in cold-responsive networks. *ZmLOX2* was approximately 70-fold upregulated in response to drought stress [108]. Expression pattern in foxtail millet contrasted with rice for salt and drought condition [40]. Based on expression intensity, upregulation was observed for salt stress and under drought stress, downregulation was observed for *SiLOX2*, *SiLOX6*, *SiLOX8* and *SiLOX9*. In cotton [109], most *LOX* genes are associated with heat and salt stress; however, *GhLOX18* was exclusively induced under cold stress, whereas some *GhLOX* genes exhibited altered expression in response to heat. Additionally, *CmLOX10* of oriental melon

significantly increases drought tolerance by mediating a JA biosynthesis pathway while only *CmLOX13* was suppressed by high temperature [27,41]. *CaLOX1* of pepper showed strong tolerance under salinity and drought [23]. Under combined drought-salt stress, upregulation of sesame *SiLOX5* was evident [107]. Overall, drought and cold emerged as the most potent inducers of *OsLOXs* gene expression, while salt, saline-alkalinity, heat and IAA decreased expression. These findings suggest that *OsLOXs* are differentially regulated by abiotic stresses, with certain genes playing key roles in drought and cold stress adaptation, whereas others show stress-specific and time-dependent regulation.

Conclusion

This study presents a thorough characterization of Lipoxygenase gene family in rice and their expression profiles under a variety of abiotic stress conditions. A total of twelve *OsLOX* members in rice were identified and categorized into three subfamilies according to structural similarities. These genes are located on seven different chromosomes and distinct conserved motifs are present. Through synteny analysis it was observed that genes underwent significant purifying selection and orthologous gene pairs evolved through segmental duplication. Notably, cis-regulatory elements analysis linked these elements to light, biotic and abiotic stress tolerance, hormonal regulation, cellular developments and core promoter associated elements. miRNA analysis helps understand the function of miRNA-modulated *OsLOX* activity. Gene ontology analysis showed the functional activity of *OsLOXs* in different physiological processes, whereas

1096 PPI analysis identified homologous proteins with Arabidopsis signaling
1097 milieu. Expression profiling through RNA-seq data demonstrated *OsLOXs*
1098 tissue-specific expression and revealed the function of *OsLOXs* in response
1099 to various biotic and abiotic stresses. Further, the *OsLOXs* expression
1100 profiling under abiotic stress treatments was evaluated by RT-qPCR data
1101 and it revealed time-dependent differential regulation of *OsLOX* genes,
1102 with drought and cold stress consistently inducing upregulation. In
1103 contrast, salt, saline-alkalinity, heat, and IAA treatments predominantly
1104 triggered sustained downregulation, with only a few transcripts showing
1105 transient or delayed induction. These findings expand the comprehension
1106 of the *LOX* gene family, establish a basis for future functional
1107 investigations of the *OsLOX* gene family and thereby offer molecular
1108 insights for enhancing rice resilience through genetic improvement and
1109 stress-tolerant variety development.

1110 **Declarations**

1111 **Ethics approval and consent to participate**

1112 Plant materials (*Oryza sativa* L.) used in this article were obtained from
1113 the Bangladesh Rice Research Institute (BRRI), Gazipur, Bangladesh. All
1114 plant materials were provided free of charge and maintained in
1115 accordance with the international guidelines. This article does not contain
1116 any studies on human participants or animals and does not involve any
1117 endangered or protected species.

1118 **Consent for publication**

1119 All authors agreed to publish.

1120 **Availability of data and materials**

1121 Data will be made available upon request.

1122 **Competing interests**

1123 The authors declare that they have no competing interests.

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1127 **Authors' contributions**

1128 **ADT** and **MNH** contributed toward conceptualization and experimental
1129 design of this research. **ADT** performed data curation, software handling,
1130 data analysis and interpretation, laboratory study, writing and preparing
1131 the original draft. **MNH** and **JFR** handled software, investigation, and
1132 manuscript revision. **MHH** and **AC** conducted investigation and
1133 validation. **SHP** supervised the project, provided scientific direction,
1134 edited and revised the final manuscript. All authors reviewed and
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1142 **Abbreviations**

1143 *LOXs*, Lipoxygenase; PUFAs, poly unsaturated fatty acids; MeJA, methyl
1144 jasmonate; CREs, cis-regulatory elements; RNA-Seq data, RNA (cDNA)
1145 Sequencing data; FPKM, Fragment Per Kilobase of exon per Million
1146 mapped read; PEG, polyethylene glycol; RT-qPCR, quantitative real time
1147 polymerase chain reaction.

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List of Figures

Figure 1. Multiple sequence alignment of OsLOX proteins. The PLAT/LH2 and Lipoxygenase domain are distinguished by bold lines at the N and C termini, respectively. Amino acids (identical or similar) are also shaded.

Figure 2. Phylogenetic relationship of LOX proteins in *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays*, *Brassica rapa*, *Sorgum bicolor*, *Setaria italica* and *Glycine max*. Maximum likelihood approach with 1000 bootstrap values were employed to generate the tree in MEGA11 software. The subfamilies 9-LOX, 13-LOX Type I, and 13-LOX Type II are represented by the violet, yellow, and blue clades, respectively. Red Color highlighted the OsLOX proteins.

Figure 3. Diagrammatic representation of conserved motifs and gene structure in twelve rice *OsLOXs*. a) Black lines denote introns, while orange boxes indicate exons. The lengths of exons and introns can be inferred using the scale provided below. b) 15 distinct colored boxes

illustrate the conserved motifs of OsLOXs. The legends below denote the protein sequence consensus for the respective motifs.

Figure 4. Chromosomal distribution of *LOX* genes throughout rice genome. MapChart software was used to generate the map. At the top, chromosome number was indicated with sequential numbering, and the centromere location was indicated by "C." Left side of the figure shows referencing scale for comparing the location of each gene.

Figure 5. Duplication events of *OsLOXs* within the genome of *Oryza sativa* and synteny analysis of *OsLOXs* between two different genomes. a) The intraspecific collinearity analysis of the replicative *OsLOXs* in rice. The red lines denote segmental duplications, whereas the blue lines signify tandem duplicates. b) Interspecific synteny analysis of *LOX* genes of *Oryza sativa*, *Arabidopsis thaliana*, and *Zea mays*. The red lines denote collinear pairings in various species. The chromosomes of different species were indicated by different color bars. The grey lines in the background denote the collinear blocks of the entire genome across various species.

Figure 6. Identified cis-regulatory elements within *OsLOXs* promoter region. The scale at the end indicates the upstream nucleotides of the translation initiation site. The colorful boxes on the right represent functions linked to distinct cis-acting elements of the corresponding genes.

Figure 7. Recognition of potential miRNAs that target *OsLOXs*. Cytoscape was employed to create a graphic depiction of the interaction between miRNA and *OsLOXs*. The miRNAs are depicted in green boxes, the *OsLOXs* are represented by elliptical shapes of various hues, and the regulatory link is indicated by arrows.

Figure 8. Gene Ontology enrichment analysis of differentially expressed genes (DEGs) associated with *OsLOXs*. The classification of *OsLOX* gene functions is displayed on the right side of the circos plot. The quantity of genes associated with a specific GO ID, the total number of genes, differential genes, expected values, and rich factors are presented in a unique color scheme. The scaling of the $-\log_{10}$ (p-value) is represented in three separate colors: red, yellow and green.

Figure 9. The distribution of predicted phosphorylation sites of OsLOX proteins. Vertical red, green, and blue lines in the middle graph reflect projected phosphorylation site scores for serine, threonine, and tyrosine, respectively. The average of the anticipated score threshold of 0.5 is shown by the horizontal pink line.

Figure 10. Analysis of the protein-protein interaction network and functional pathways associated with the *OsLOX* gene family. a) PPI network of the *OsLOX* family; b) KEGG enrichment study of proteins within PPI nodes.

Figure 11. Secondary structure analysis of the OsLOXs. The structure was generated employing the STRIDE tool, and the membrane-spanning motif was found by the DeepTMHMM v2.0 server. The transmembrane helix is represented by the black box. The icons for secondary structure legends are displayed in the lower-right corner.

Figure 12. Predicted 3D structures of OsLOX proteins represented in a gradient of rainbow colors from N (blue) to C terminus (red) utilizing PyMOL v3.1 software.

1686 **Figure 13.** Heatmap depicting the expression profiling of *OsLOXs* in rice
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1690 low expression. Abbreviations: 7 DBH - seven days before heading; 7 DAF
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1692 days after germination; 5 DAP - five days after pollination.

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1696 GraphPad Prism 10.5.0 software. The right-side scale of the heatmap
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1699 moderate expression levels, respectively.

1700 **Figure 15.** Heatmap illustrating the expression profiling of *OsLOXs* in
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1704 heatmap illustrates relative expression values based on the log₂ FPKM
1705 values. The colors red, green, and black represent high, low, and moderate
1706 expression levels, respectively.

1707 **Figure 16.** Relative expression analysis of *OsLOXs* in response to a variety
1708 of abiotic stress conditions. The Y-axis represents the relative expression
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1710 The X-axis represents the various abiotic stress conditions under which the
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1715 different means were annotated with a corresponding number of asterisks
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